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Letters to the Editors

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ᗺE DEPENDENCE OF THE SPECIFIC ACTIVITY OF UREASE UPON THE APPARENT ABSOLUTE ENZYME CONCENTRATION

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If the actıvıty of an enzyme preparatıon ıs determıned under condıtıons which a further ıncrease ın substrate concentratıon ıs without demon- ɾable effect, all other factors beıng held constant, ıt ıs ordınarıly assumed at the specıfic actıvıty of the enzyme, expressed ın terms of arbıtrary ·'s per unıt weıght of the enzyme, ıs ındependent of the absolute enzyme ıcentratıon (1, 2) However, wıth urease solutıons stabılızed wıth hy- ̀gen sulfide or cysteıne (1) we have observed that the specıfic actıvıty ı gıven urease preparatıon, when determıned under the above condıtıons, reases wıth decreasıng apparent enzyme concentratıon over a wıde range concentratıons and that thıs ıncrease ın specıfic actıvıty proceeds wıth ɑcasurable velocıty at temperatures above 15° Thıs phenomenon was erved wıth crude ureasc preparatıons, such as jack bean meal, and wıth ɔ, three, and seven tımes recrystallızed urease Sınce lıttle or no dıffer- e was observed ın the behavıor of three and seven tımes recrystallızed ·ase, the data presented ın thıs paper are lımıted to those obtaıned wıth ıce recrystallızed preparatıons Urease actıvıty was determıned by a ɔdıficatıon of the procedure descrıbed by Van Slyke and Cullen (3) The ·ecısıon of the modıfied procedure was ±2 to 3 per cent

EXPERIMENTAL

Determınatıon of Urease Actıvıty

Reagents—The buffer solutıon, 0 1 M ın phosphate adjusted to pH 7 0, ı ın all experıments was prepared from dıpotassıum hydrogen phosphate ɔotassıum dıhydrogen phosphate The 1 0 M solutıon of urea was pre- ɜd daıly ın order to mınımıze the effects of bacterıal contamınatıon ɑe crystallıne urease was prepared from jack bean meal by the method ı Dounce (4), all operatıons subsequent to the ınıtıal extractıon beıng ırrıed out at 5° Thrıce recrystallızed urease from 200 gm of meal was

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dissolved in 3 to 5 ml of water, 1 per cent saturated with hydrogen sulfide, and this stock solution kept at 5° prior to its use Hydrogen sulfide solutions were prepared daily by appropriate dilution of a solution saturated at 0° Redistilled water was used in all cases

Procedure—Clean 18 × 150 mm reaction tubes were charged with 2 0 ml of buffer solution and 1 0 ml of 1 0 M urea solution (or standard ammonium sulfate solution containing 100 γ of ammonia nitrogen per ml) and placed in a constant temperature bath at either 25° or 15° When thermal equilibrium had been attained, the enzyme solution was added (usually 1 00 or 0 79 ml) and the time noted After the desired time interval had elapsed (usually 2 minutes), 0 5 ml of 1 0 M sulfuric acid was added to each of the tubes, the latter were shaken and placed in an ice bath Exactly 10 ml of 0 01 M sulfuric acid were placed in each 18 × 150 mm absorption tube and the tubes fitted with rubber stoppers, each bearing a 4 mm glass inlet tube and a capillary type critical orifice designed to permit an air flow of 300 to 400 ml per minute To each of the reaction tubes in the ice bath were added 3 to 4 gm of anhydrous potassium carbonate, and the tubes were fitted with rubber stoppers, each bearing a 4 mm inlet tube as well as a 4 mm U-tube serving to connect the reaction tube with the absorption tube Each reaction tube was connected to an absorption tube with a short length of rubber tubing and placed in a 55° bath, the inlet and outlet tubes connected to manifolds, and the aeration started After 20 minutes each reaction tube was disconnected and shaken so as to wash down the sides of the tube with carbonate solution After this process, the tubes were reconnected and allowed to aerate for a second period of 20 minutes, when the washing process was repeated After a final 20 minutes aeration the absorption tubes were disconnected and placed in an ice bath To the chilled contents of each of the absorption tubes was added 1 0 ml of Nessler's reagent (5), the tubes were removed from the ice bath, and after 10 minutes the intensity of the color measured in a Klett colorimeter With the apparatus at our disposal it was possible to run eleven determinations simultaneously and in every such series two or more determinations were blanks in which either the urea solution or the urease solution was replaced by an equal volume of water or standard ammonium sulfate solution For precise results such blank determinations were found to be necessary The conversion of colorimeter readings to micromoles of ammonia was based upon a series of determinations of the ammonia recoverable in the range of 10 to 200 γ of ammonia nitrogen from a standard solution of ammonium sulfate The specific activity was calculated from the relation specific activity $= 10x/ta$, where $x =$ the number of micromoles of ammonia liberated, $t =$ the time in minutes during which hydrolysis occurred, and $a =$ the micrograms of protein nitrogen present in the solution

Dilution Experiments

Procedure—Relatively concentrated solutions of thrice recrystallized urease in water 1 per cent saturated with hydrogen sulfide, *i e*, containing 300 to 1500 γ of protein nitrogen per ml, were diluted with solutions of hydrogen sulfide or cysteine previously adjusted to pH 7 2 to 7 5 by the addition of anhydrous potassium carbonate[1] The specific activity of urease in these solutions was then determined as a function of time, zero time being taken as the time of mixing In those experiments in which a stepwise dilution technique was employed the solutions were allowed to stand at 25° for 2 hours after the initial dilution A 1 10 6000 stepwise dilution is defined as an initial 1 10 dilution which has subsequently been diluted to effect a final over-all dilution of 1 6000 The data obtained in these experiments are given in Tables I to III

Determination of Michaelis Constants

Procedure—The kinetics of the hydrolysis of urea by thrice recrystallized urease were studied at 25° and pH 7 0 The activity of the urease solutions was determined as described previously and the rate of determinations were made at nine different urea concentrations, *i e*, 0 004, 0 005, 0 006, 0 007, 0 008, 0 010, 0 015, 0 025, and 0 250 M urea The data so obtained were found to obey the Michaelis-Menten equation, $v = VS/(Km + S)$, where v = the rate of hydrolysis at a urea concentration S and V = the maximum or limiting rate of hydrolysis over the concentration range of substrate studied In practice it was found convenient to follow the suggestion of Lineweaver and Burke (6) and to transform the original Michaelis equation into its linear form $1/v = Km/VS + 1/V$ for evaluation of the data It should be noted that the concentration of urea corresponding to a given rate was always taken as the average urea concentration obtaining during the determination of that rate

DISCUSSION

The dependence of the specific activity of urease upon the apparent enzyme concentration in systems containing hydrogen sulfide was first observed when relatively concentrated solutions of urease in water containing hydrogen sulfide were diluted approximately a thousand fold with the same solvent[2] It was further observed[2] that in the absence of hydrogen sulfide rapid inactivation of the urease occurred and that the effect observed with solutions containing hydrogen sulfide could be obviated by the presence of silver ion In earlier experiments it was not appreciated that urease is

[1] The pH of water 1 to 10 per cent saturated with hydrogen sulfide was found to vary between 5 5 and 4 5

[2] Unpublished experiments of the authors

slowly inactivated at pH 4 5 to 5 5 in solutions containing hydrogen sulfide However, it was found that this inactivation could be minimized or suppressed by maintaining the systems at approximately pH 7 at all times

The data presented in Columns 2 and 3 of Table I are typical of those observed when relatively concentrated solutions of urease are diluted at 25° and pH 7 with water containing hydrogen sulfide and the specific activity of the urease in these solutions determined as a function of time, with zero time as the time of mixing The initial values are not particularly accurate, since the actual determination of urease activity requires a minimum of 2 minutes if precise results are to be obtained Nevertheless the data

TABLE I

*Dilution of Urease Solutions with Water 1 Per Cent Saturated with Hydrogen Sulfide at 25° and pH 7 2 to 7 5**

Time after dilution	Specific activity at 25°, pH 7 0†					
	1 6000 dilution		1 4 6000 dilution		1 10 6000 dilution	
	Experiment I	Experiment II	Experiment I	Experiment II	Experiment I	Experiment II
(1)	(2)	(3)	(4)	(5)	(6)	(7)
min						
2	136	131	138‡	146‡	156	148
10	141	134‡	145	151	161	153
30	149	139	156‡	154	168	154
70	169	163	186	170‡	181	163
120	186	175	207	186	196	184
180	198	191‡	218		203‡	
240	210	207	228	204	210‡	204
300	220		228		217	

* The original urease solution contained 1365 γ of protein nitrogen per ml

† Average of duplicate determinations, agreeing within ±2 per cent

‡ Obtained from a smooth curve through other points of the series

in Columns 2 and 3 of Table I clearly illustrate that the specific activity of the urease in these solutions increases with time

The increase in the specific activity of urease upon dilution noted above was observed when urease solutions containing 1365 γ of protein nitrogen per ml were diluted to give solutions containing approximately 0 23 γ of protein nitrogen per ml In order to determine whether this effect could be observed with dilutions of lower order dilutions of 1 4 and 1 10 were employed, these solutions were allowed to stand sufficiently long to attain equilibrium, and then diluted uniformly to the point at which it was possible to determine the specific activity of the urease contained therein rea-

sonably accurately As before, the specific activity of the urease in the final solutions was determined as a function of time (Table I, Columns 4 to 7) It will be noted that in every case the specific activity increased with time and reached, within experimental error, the same maximum limiting value Further, it is clear from these data that the initial 1 4 and 1 10 dilutions were effective in diminishing the percentage change in specific activity observable upon the final 1 6000 dilution, and one may conclude not only that dilutions of low order were attended by changes in specific activity, but also that at equilibrium and with relatively high concentrations of urease the specific activity of urease is a function of the enzyme concentration if the latter is taken as being equivalent to the amount of protein nitrogen present in solution

TABLE II

*Dilution of Urease Solutions with Water 1 Per Cent Saturated with Hydrogen Sulfide at 15° and pH 7 3**

Time after dilution	Specific activity at 15 , pH 7 0, after 1 4000 dilution	
	Experiment I	Experiment II
min		
2	88	83
15	88	
45	86	88
60	91	93

* The original urease solution contained 1365 γ of protein nitrogen per ml

An attempt was made to estimate the magnitude of the temperature coefficient of the above reaction by measuring the change in the specific activity of urease brought about by a 1 4000 dilution of a relatively concentrated urease solution at 15° and 25° and at pH 7 3 Unfortunately the data obtained in these experiments (Table II) did not justify the calculation of a value for the temperature coefficient However, they do provide evidence that the reaction is dependent on temperature

The above observations on the behavior of urease in solutions containing hydrogen sulfide suggested the desirability of investigating the effects observable with another so called stabilizing agent In Table III are presented data which were obtained in preliminary experiments in which cysteine was used instead of hydrogen sulfide While these data are not as extensive as those available for hydrogen sulfide, it is clear that in both systems similar, but not necessarily identical, reactions are operative

The Michaelis constant (1, 2, 7) of an enzyme is often taken as a char-

acteristic property of the enzyme, though it is recognized (7) that the constant may be dependent upon temperature and pH[3] Using crude preparations, Van Slyke and Cullen (3) and Ambros and Munch (8) obtained data which give values of 0 011 M urea at 20° and pH 7 and 0 0082 M urea at 50° and pH 7 6 for the Michaelis constant of urease With urease solutions containing 0 065 to 0 095 γ of protein nitrogen per ml, which had been prepared by dilution with water 1 per cent saturated with hydrogen sulfide and allowed to stand at 25° for 3 to 4 hours, values for the Michaelis constant of five different urease preparations, obtained from two different lots of jack bean meal, were found to be 0 0098, 0 0116, 0 0098, 0 0112, and 0 0103 M urea at 25° and pH 7 0 respectively The variation in the Michaelis constant noted above, i e 0 0107 ± 0 0009, was somewhat greater than that observed, i e 0 0106 ± 0 0003, when the constant of a given urease preparation was redetermined at intervals over a period of several months

TABLE III

*Dilution of Urease Solutions with Solutions of Cysteine at 25° and pH 7 0**

Time after dilution	Specific activity at 25°, pH 7 0†			
	0 01 M cysteine	0 002 M cysteine		
	1 5000 dilution	1 5000 dilution	1 4 5000 dilution	1 10 5000 dilution
min				
2	119	137	123	98
10	123	141	129	103
30	134	150	136	109
70	154	169	152	121
120	179	183	166	130
180	188	190	171	132‡
240	198	193	175	132
360	198	176	189	150

* The original urease solution contained 1365 γ of protein nitrogen per ml

† Average of duplicate determinations, agreeing within ±2 per cent

‡ Obtained from a smooth curve through other points of the series

The fact that a substantial increase in the specific activity of urease is observed when a relatively concentrated solution of urease in water containing hydrogen sulfide or cysteine is diluted with the same solvent appears to preclude the possibility that the effect observed is simply an activation of urease by hydrogen sulfide or cysteine (9) An alternative explanation may be that the urease molecule dissociates into smaller units upon dilution and that this process is accompanied by an increase in the number of re-

[3] Ionic strength and the nature of the buffer may also be important variables

active sites, the hydrogen sulfide or cysteine merely serving to prevent inactivation A second explanation may be that the crystalline urease preparations are contaminated with a naturally occurring inhibitor, which is not removed by the repeated recrystallization of urease from relatively concentrated solutions, and that the urease-inhibitor complex dissociates in dilute solutions, the degree of dissociation being a function, within limits, of the degree of dilution Although there are insufficient data to determine whether all of the above hypotheses are operative or whether any one should be completely excluded, it is clear that the observed effect must be taken into account if studies on urease action are to be properly evaluated

SUMMARY

It has been observed that the specific activity of urease in solutions containing hydrogen sulfide, or cysteine, and expressed in terms of arbitrary units of urease activity per unit weight of enzyme taken as protein nitrogen is dependent, within limits, upon the apparent enzyme concentration The Michaelis constants of several urease preparations have been determined at 25° and pH 7 0 under conditions minimizing the above phenomenon

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STUDIES ON DIPEPTIDASES

II SOME PROPERTIES OF THE GLYCYL L-LEUCINE DIPEPTIDASES OF ANIMAL TISSUES*

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One of the main problems connected with the study of peptidase activities concerns the individuality and specificity of the enzymes which are responsible for the hydrolysis of the many peptides which can be split by animal tissues Thus far, only a few exopeptidases have been extensively studied, and the problem of classification of these activities demands considerable investigation prior to the actual isolation of these enzymes The apparent instability of most of the enzymes which act on simple peptides has rendered the task of purification quite difficult However, since it has become clear that many of the peptidases require the presence of heavy metals for full activity, the position is now somewhat better for establishing the conditions for the study of these enzymes

The present investigation deals with the enzymes which are responsible for the hydrolysis of glycyl-L-leucine (GL) as these are found in hog intestinal mucosa, human uterus, and in rat and rabbit muscle These enzymes all exhibit the specificity characteristics of dipeptidases, since the peptide derivatives which do not contain both free amino and free carboxyl groups are resistant to hydrolysis Like the glycylglycine dipeptidase discussed earlier (2), the activity towards GL does not appear to parallel the hydrolysis of any other peptide which has been studied The GL-splitting enzymes are, therefore, tentatively classified as specific glycyl-L-leucine dipeptidases

Effect of Metal Ions

It has been found that the GL-splitting enzymes of different tissues may be quite specific in the type of metal which is required for activation For example, with extracts of hog intestinal mucosa the hydrolysis of GL is activated by Mn^{++}, while the enzyme of human uterus is specifically ac-

* This investigation was supported by a grant from the United States Public Health Service A report on a part of this work was presented at a meeting of the American Society of Biological Chemists at Atlantic City (March, 1948) (1) For the first paper in this series, see (2)

tivated by Zn^{++} A complicating factor is that all of these enzymes are inhibited by Ca^{++} Crude extracts of the tissues seldom exhibit their maximal activity, except in the presence of phosphate The behavior of the enzymes in the presence of divalent metal ions will be discussed separately for the different tissues

Human Uterus—Fig 1 shows some of the data obtained with an aqueous extract of an acetone powder prepared from a filtered water extract of human uterus (3) In the presence of both Zn^{++} and phosphate, a maximal rate of splitting is found, and the reaction proceeds with the kinetics of a

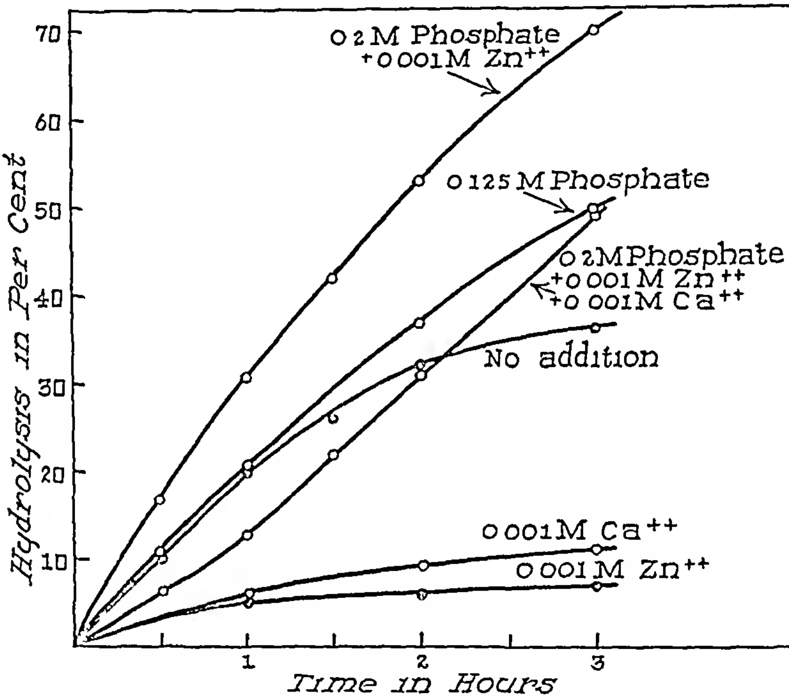

FIG 1 Effect of metal ions and phosphate on the hydrolysis of glycyl-L-leucine by an enzyme preparation from human uterus The experiments were performed at 40° at pH 7 8 to 8 0 at an enzyme concentration of 0 0102 mg of protein N per cc

first order reaction In the absence of phosphate (no buffer added), the hydrolysis is inhibited by Ca^{++} and, more surprisingly, by Zn^{++} also Addition of phosphate alone gives a gradually increasing rate as compared to the control Simultaneous addition of phosphate, Ca^{++}, and Zn^{++} gradually reverses the inhibitory effect of Ca^{++}

While phosphate is fairly specific in allowing the maximal activity of the enzyme to be manifest, citrate is partially effective The lower three experiments in Fig 2 were conducted in the presence of 0 02 M veronal buffer It is apparent, however, that highest activity is obtained only with the combination of Zn^{++} and phosphate, and that citrate is not completely

effective in reversing the inhibitory effect which Zn^{++} has in the absence of phosphate The GL dipeptidase of uterus is not activated by other metal

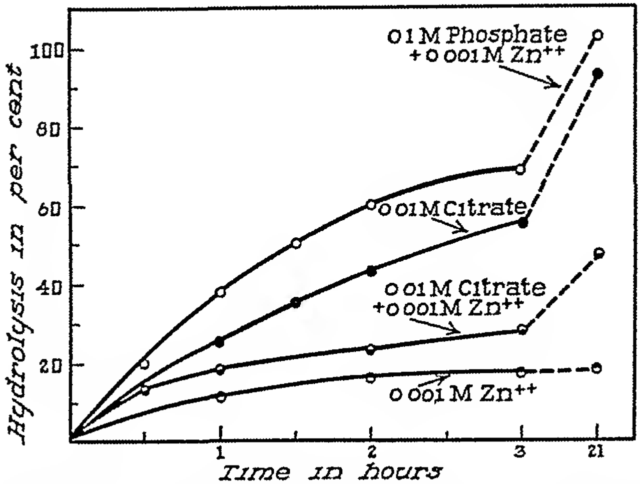

FIG 2 Effect of citrate on the hydrolysis of glycyl-L-leucine by an enzyme from human uterus The experiments were performed at 40° at pH 7 8 to 8 0 at an enzyme concentration of 0 0102 mg of protein N per cc The three experiments at the bottom of the graph were carried out in the presence of 0 02 M veronal buffer

TABLE I

Effect of Metals on Hydrolysis of Glycyl-L-leucine

The experiments were performed at 40° and at pH 7 7 to 7 8 The metals were present at a concentration of 0 001 M The preparations of hog intestinal mucosa and rat muscle were filtered aqueous extracts The preparations from rabbit muscle and human uterus were made from acetone powders obtained from the water extracts of these tissues

Tissue	Buffer	Protein N per cc test solution	Time	Hydrolysis, per cent					
				No metal	Mn^{++}	Mg^{++}	Zn^{++}	Co^{++}	Fe^{++}
		mg	*hrs*						
Hog intestinal mucosa	0 02 M veronal	0 52	0 5	76	39	45	32	20	41
			1	106	49	62	46	37	71
Rat muscle	0 02 " "	0 47	1	69	40	67	21	41	72
	0 1 " phosphate		1	72	51	79	83	51	63
Rabbit muscle	0 02 " veronal	0 43	5	22	55	24	16	51	
			24	69	103	74	40	103	
Human uterus	0 1 " phosphate	0 0069	2	31	19	27	45	14	

ions which have been tested In fact, inhibition is produced by Mg^{++} or Co^{++} (Table I)

It should be noted that, in a few instances, Zn^{++} activated the enzyme even in the presence of veronal buffer (3) However, for completely reproducible results and for maximal activities, it has been found desirable to use phosphate buffer

Rat Muscle—Crude aqueous extracts of rat muscle contain a GL dipeptidase which is very similar to that of human uterus In veronal buffer, the enzyme is inhibited by 0 001 M Mn^{++}, Zn^{++}, Co^{++}, and Ca^{++} Mg^{++} and Fe^{++} have no effect In the presence of 0 1 M phosphate buffer, the enzyme is strongly activated by Zn^{++}, slightly activated by Mg^{++}, and is inhibited by Mn^{++} and Co^{++} (Table I)

Rabbit Muscle—Aqueous extracts of fresh muscle or of an acetone-dried powder show an Mn^{++} or Co^{++} activation of GL hydrolysis in the presence of veronal or phosphate Other ions are inhibitory (Zn^{++}), or show no effect whatsoever (Mg^{++})

Hog Intestinal Mucosa—A centrifuged aqueous extract of the mucosa displays no effect of activation, and the hydrolysis of GL is inhibited by 0 001 M Mn^{++}, Co^{++}, Mg^{++}, Zn^{++}, and Fe^{++} (Table I) A stable preparation may be obtained from the crude extract by collecting the precipitate which forms between 0 4 and 0 8 saturation with ammonium sulfate The dialyzed and filtered solution shows a negligible activity in the absence of metal ions, but is strongly and specifically activated by Mn^{++} This Preparation A was used for subsequent studies of kinetics and activation behavior

Kinetics

It is evident from Table II that Preparation A of hog intestinal mucosa is strongly activated by Mn^{++} If the enzyme is not preincubated with Mn^{++}, a considerable lag period is found during which the rate of hydrolysis of GL gradually increases Thus, the reaction between Mn^{++} and protein to form an active enzyme is a time reaction like that previously described for other peptidases (3–5) After incubation of the protein with Mn^{++} for 3 hours, the hydrolysis of GL proceeds with the kinetics of a first order reaction

When activation is produced by the specific metal with a crude tissue extract, no lag period is shown in the hydrolysis of the substrate, and the hydrolysis of GL proceeds as a first order reaction For the different extracts, the velocity constants K are proportional to the enzyme concentration Table III shows that with the uterine extract the proteolytic coefficient C is constant over a wide range in the presence of Zn^{++} and phosphate buffer It should be noted that, for these experiments, satisfactory kinetic measurements can be made with as little as 0 00346 mg of protein N per cc of test solution It is evident that the GL dipeptidase of uterine tissue is extremely active

The experiments with the extract of rat muscle show satisfactory first order kinetics in the presence of Zn^{++} and phosphate It is apparent from Table III that even in the presence of Mn^{++} and veronal, where the full activity of the enzyme is not manifest, K is proportional to the enzyme concentration

The activation of hog mucosal Preparation A was studied at different Mn^{++} concentrations A constant amount of enzyme (0 514 mg of protein per cc) was incubated with Mn^{++} for 3 hours and an aliquot was then added to the buffered substrate solution The first order velocity con-

TABLE II

Hydrolysis of Glycyl-L-leucine by Hog Intestinal Mucosa

The experiments were performed at 40° in 0 1 M phosphate buffer at pH 7 8 C is the proteolytic coefficient defined as the first order velocity constant K at enzyme concentration E, expressed in mg of protein N per cc where $C = K/E$ The enzyme was Preparation A

Conditions	Enzyme concentration in test solution	Time	Hydrolysis	C
	mg protein N per cc	*hrs*	*per cent*	
No metal added	0 103	3	1	
Contained 0 001 M Mn^{++}, no prior incubation	0 103	0 5	1	
		1	19	
		1 5	29	
		2	48	
Contained 0 003 M Mn^{++} in test solution after prior incubation for 3 hrs of 0 02 M $MnCl_2$ with enzyme	0 072	0 5	17	0 036
		1	32	0 038
		1 5	38	0 032
		2	51	0 035
		2 5	58	0 035
		3	64	0 035

stants are shown as a function of log Mn^{++} concentration in Fig 3 The highest Mn^{++} concentration increases the rate of hydrolysis about 27-fold The course of the activation is that of the typical mass law form previously described for other peptidases (2, 6) The apparent dissociation constant of the metal-protein complex is $1\ 8 \times 10^{-4}$ This value must be taken only as an approximation, since the experiments were performed in the presence of phosphate which may bind some of the metal The true concentration of free Mn^{++} may be lower than that of the total Mn^{++} present in the test solutions

Specificity

Although the hydrolysis of dipeptides has frequently been ascribed to dipeptidases, evidence has seldom been presented in support of this view

Table III

Effect of Enzyme Concentration on Hydrolysis of Glycyl-L-leucine

The measurements were performed with a crude filtered aqueous extract of rat muscle, with an aqueous extract of an acetone-dried powder of human uterus, and with a crude aqueous extract of hog intestinal mucosa The tests with uterine extract were made at 40° in the presence of 0 001 M Zn^{++} and 0 15 M phosphate buffer at pH 7 8 The test solutions with the muscle extract contained 0 001 M Mn^{++} and veronal buffer The experiments with the crude intestinal extract contained 0 04 M veronal and no added metal ion The proteolytic coefficient $C = K/E$, where K is the first order velocity constant for the enzyme concentration E given in mg of protein N per cc of test solution

Tissue extract	Enzyme concentration	Time	Hydrolysis	$K \times 10^{-3}$	C average
	mg protein N per cc	*hrs*	*per cent*		
Human uterus	0 00346			1 04	0 30
	0 00692			1 98	0 29
	0 0102			3 04	0 30
	0 0138			4 0	0 29
				4 4	0 32
	0 0277			8 3	0 30
	0 0415			12 6	0 30
				11 9	0 29
	0 0692			19	0 27
				20	0 29
Rat muscle	0 109	7	28	0 34	
		24	73	0 39	0 0034
	0 163	3	18	0 46	
		5	33	0 57	
		6	39	0 60	
		7	46	0 64	0 0035
	0 218	2	18	0 73	
		4 5	35	0 70	
		5 5	43	0 75	
		7	54	0 81	0 0034
	0 272	2	22	0 89	
		3	33	0 95	
		4 5	46	0 97	
		5 5	54	0 99	0 0035
	0 408	1 5	29	1 67	
		2	36	1 59	
		2 5	42	1 55	
		3	47	1 55	
		4	57	1 55	0 0039
Hog intestinal mucosa	0 104			2 5	0 024
	0 26			6 4	0 025
	0 52			12 0	0 023

Maschmann (7) has suggested that the hydrolysis of certain dipeptides, including GL, is performed by substrate-specific enzymes However,

tests must be performed with appropriately substituted derivatives in order to determine the specificity of the enzyme involved in each instance. Thus, while the hydrolysis of glycylglycine is apparently due to a specific dipeptidase (2), the hydrolysis of L-leucylglycine is performed by an enzyme which has the specificity of an aminopeptidase (5, 8).

In Table IV are given some data on the specificity of the extracts of human uterus and of rat muscle. The uterine extracts show no splitting of carbobenzoxyglycyl-L-leucine, indicating that a carboxypeptidase is not involved and that a free amino group is essential. The slow hydrolysis of carbobenzoxyglycyl-L-leucinamide is not activated by Zn^{++} and requires at

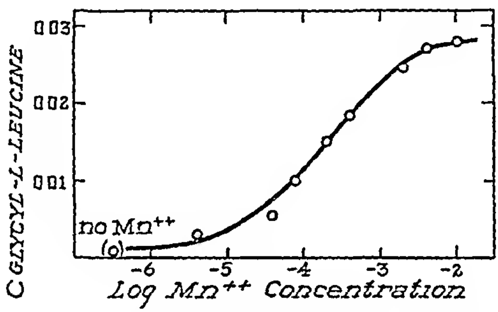

FIG. 3. Effect of concentration of Mn^{++} (in moles per liter) on the proteolytic coefficient for hydrolysis of glycyl-L-leucine by Preparation A of hog intestinal mucosa. For each experimental point, the enzyme (0.514 mg. of protein N per cc.) was incubated with Mn^{++} for 3 hours at 40° in the presence of 0.3 M phosphate buffer at pH 7.7. An aliquot was then added to the substrate solution buffered with 0.1 M phosphate at pH 7.8. The concentration of enzyme in each test solution was one-fifth of the incubation value. Correspondingly, the plotted Mn^{++} values are those for the test solutions, the incubation mixtures contained five times as much. Each plotted point is the average of two independent kinetic runs made on successive days.

least 10 times the amount of enzyme necessary for hydrolysis of the free peptide. This hydrolysis must therefore be due to an endopeptidase of a different character.

With the uterine enzyme at a concentration of 0.082 mg. per cc., the hydrolysis of GL is nearly complete in 1 hour, while the hydrolysis of glycyl-L-leucinamide (GLA) proceeds quite slowly. With a 5-fold increase in enzyme concentration, it was noted that the initial hydrolysis of GLA is slightly inhibited by Zn^{++}, but is strongly activated by Mn^{++}. Moreover, in the presence of Mn^{++} both of the sensitive peptide bonds are hydrolyzed. It has recently been found that a highly purified preparation of leucine aminopeptidase rapidly hydrolyzes the amide bond of GLA to form GL and ammonia.[1] Since the aminopeptidase which is activated by Mn^{++} is present in all crude tissue extracts, it introduces a complicating factor

[1] Smith, E. L., and Slonim, N. B., to be published.

It appears that the initial hydrolysis of GLA must take place at the amide bond by the action of the aminopeptidase, this is followed by the scission of the formed GL by the dipeptidase

$$\text{Glycyl-L-leucinamide} \xrightarrow[\text{Mn}^{++}]{\text{Leucine aminopeptidase}} \text{Glycyl-L-leucine} + \text{NH}_3$$

$$\text{Glycyl-L-leucine} \xrightarrow[\text{Zn}^{++}]{\text{Glycyl-L-leucine dipeptidase}} \text{Glycine} + \text{leucine}$$

TABLE IV

Specificity of Glycyl-L-leucine Dipeptidase

The experiments were performed at 40° in 0.1 M phosphate buffer at pH 7.8 with a substrate concentration of 0.05 M. Carbobenzoxyglycyl-L-leucinamide was only partially in solution. Hydrolysis is expressed as 100 per cent for the complete splitting of one peptide bond.

Tissue	Substrate	Enzyme concentration	Metal	Time	Hydrolysis
		mg protein N per cc		*hrs*	*per cent*
Human uterus	Glycyl-L-leucine	0.082	0.001 M Zn^{++}	1	91
	Carbobenzoxyglycyl-L-leucine	0.82	0.001 " "	22	4
	Carbobenzoxyglycyl-L-leucinamide	0.82	0.001 " "	5	28
				22	57
	Glycyl-L-leucinamide	0.082	0.001 " "	3	3
				20	16
	"	0.41	0.001 " "	1	9
				3	16
				20	75
	"	0.41	None	1	4
				3	13
				20	103*
	"	0.41	0.001 M Mn^{++}	1	20
				3	95
				20	165*
	L-Leucinamide	0.205	0.001 " "	3	88
Rat muscle	Glycyl-L-leucine	0.24	0.001 " Zn^{++}	3	84
			0.001 " Mn^{++}	3	49
	Carbobenzoxyglycyl-L-leucine	0.95	0.001 " Zn^{++}	23	3
	Carbobenzoxyglycyl-L-leucinamide	0.95	0.001 " "	23	10
			None	23	27
	Glycyl-L-leucinamide	0.24	0.001 M Zn^{++}	3	16
				20	44
	"	0.24	0.001 " Mn^{++}	3	31
				20	133*

* 100 per cent for the splitting of one peptide bond

If the initial hydrolysis were due to the dipeptidase, Zn^{++} should exert a strong activating effect Since it has not yet been possible to remove the aminopeptidase from these preparations, the question must remain open whether the enzyme which splits GL has a very slow action on GLA Nevertheless, the GL-splitting enzyme does appear to have the specificity of a dipeptidase, since its action on the substituted compounds must be very slight indeed

The crude extract of rat muscle shows a specificity similar to that of the uterine extract (Table IV) Here also, the differential effects of Zn^{++} and Mn^{++} on the splitting of GLA can be used to distinguish between the action of the Mn^{++}-activated leucine aminopeptidase present in this tissue (3) and the Zn^{++}-activated dipeptidase which acts on GL With this extract, the endopeptidase action on carbobenzoxyglycyl-L-leucinamide is inhibited by Zn^{++}

With extracts of hog intestinal mucosa and rabbit muscle, it is not possible to utilize the two metals in differentiating between the leucine aminopeptidase and the GL dipeptidase, since both of the enzymes from these tissues are activated by Mn^{++} Hence, the consecutive splitting of both peptide bonds of GLA proceeds with great rapidity in the presence of Mn^{++} Nevertheless, since the leucine aminopeptidase of hog mucosa has been obtained substantially free of the GL dipeptidase,[1] it appears legitimate to ascribe to the aminopeptidase all of the activity of the crude extract towards GLA, particularly since the purified enzyme shows this action to approximately the same relative extent as does the crude preparation

DISCUSSION

The demonstration that the hydrolysis of GL is due to a dipeptidase provides the second instance in which it has been shown that the splitting of a dipeptide is due to a specific enzyme The hydrolysis of glycylglycine has also recently been indicated to be due to a specific dipeptidase (2) Although the use of substituted compounds has provided the evidence for classifying these hydrolytic enzymes as dipeptidases, it is premature to conclude that other dipeptides are not hydrolyzed by these enzymes The fact that Zn^{++} is a specific activator for two of the enzymes which split GL immediately differentiates these from the many other enzymes which split simple dipeptides and which are not activated by this metal However, Zn^{++} has recently been reported to be the metal involved in a dehydropeptidase (8) We have found that the hydrolysis of glycyl-L-tryptophan by preparations from human uterus is also activated by Zn^{++} Nevertheless, it can be stated that the hydrolysis of GL and of glycyl-L-tryptophan is due to distinct enzymes, since the GL dipeptidase is destroyed to a much greater extent by heating to 50° for 10 minutes than is the enzyme which splits glycyl-L-tryptophan Up to the present time, we

have not been able to obtain any evidence that the dipeptidase which splits GL possesses any other type of action

The assistance of Mrs Toshiko Shimizu is gratefully acknowledged

EXPERIMENTAL

Hydrolysis was measured by the titration of liberated carboxyl groups by the method of Grassmann and Heyde (9) The substrates were present at a concentration of 0 05 M Hydrolysis is expressed as 100 per cent for the complete splitting of one peptide bond The acetone-dried preparations of human uterus and of the rat muscle extract have already been described (3) The substrates prepared were as follows glycyl-L-leucine (10) and carbobenzoxyglycyl-L-leucine (11) Carbobenzoxyglycyl-L-leucinamide and glycyl-L-leucinamide hydrochloride will be described later[1]

SUMMARY

1 The enzymes of human uterus and rat muscle which hydrolyze glycyl-L-leucine (GL) are activated by Zn^{++}, the corresponding enzymes of rabbit muscle and hog intestinal mucosa are activated by Mn^{++} Maximal activity in all cases is obtained with phosphate buffer apparently because of the binding of Ca^{++} which is an inhibitor of these enzymes

2 With activating metal and phosphate buffer, the hydrolysis of GL proceeds with the kinetics of a first order reaction, and is proportional to the enzyme concentration over a wide range

3 After precipitation and dialysis of the GL dipeptidase of hog intestinal mucosa, the activation by Mn^{++} is a time reaction The activity of the enzyme at different Mn^{++} concentrations shows a typical mass law relationship with an apparent dissociation constant of $1\ 8 \times 10^{-4}$

4 The GL-splitting enzymes possess the specificity of dipeptidases, since carbobenzoxyglycyl-L-leucine, carbobenzoxyglycyl-L-leucinamide, and glycyl-L-leucinamide are hydrolyzed either not at all or only very slowly by the dipeptidases

5 Extracts of human uterus and rat muscle contain an endopeptidase which slowly splits carbobenzoxyglycyl-L-leucinamide This hydrolysis is not activated by metal ions

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STUDIES ON DIPEPTIDASES

III HYDROLYSIS OF METHYLATED PEPTIDES, THE RÔLE OF COBALT IN THE ACTION OF GLYCYLGLYCINE DIPEPTIDASE*

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The recognition that the hydrolysis of the peptides, glycylglycine and glycyl-L-leucine, is due to specific dipeptidases (1-3) has led to a search for an explanation of why the responsible enzymes should require such extremely specific substrate configurations While a complete description is obviously not yet possible, a partial answer seems to be in the rôle that certain metals play in these hydrolytic reactions It was suggested that, in the case of glycylglycine dipeptidase, the function of Co^{++} is to act as a bridge forming a coordination compound involving the substrate, on the one hand, and the protein on the other (1) This hypothesis arose from the observation that at pH 7 6, where the enzyme is maximally active, Co^{++} coordinates much more readily with glycylglycine (GG) than it does with triglycine or glycine, as judged by the relative effect of these compounds on the absorption spectrum of Co^{++} This suggestion has now been subjected to further test in several ways First of all, it has been found that those substituted compounds which are not split by the enzyme react either weakly or not at all with Co^{++} Secondly, an additional substrate for the GG dipeptidase has been found in sarcosylglycine (SG), and this compound coordinates readily with Co^{++} From the closely parallel data of the specificity of the enzyme and the specificity of complex formation with Co^{++}, some evidence is available regarding the types of linkage involved

The observations with Co^{++} are facilitated by the characteristic absorption bands which can easily be studied, unlike the coordination of other metals with peptides in which we have observed only a change in the ultraviolet end-absorption On the other hand, certain difficulties appear in this work with Co^{++}, namely, the reversible oxygen binding and subsequent irreversible slow oxidation which may occur with some of these chelate compounds, as beautifully demonstrated by Burk and his collaborators (4) Nevertheless, that other effects may be found in the absence of the enzyme should not detract from the primary matter of whether or not coordination takes place

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Specificity of Glycylglycine Dipeptidase

It is shown in Table I that two tissue extracts rich in this dipeptidase (1) readily split SG and that this hydrolysis is strongly accelerated by Co^{++} in the same degree as the hydrolysis of GG Neither of these extracts has the slightest detectable effect on glycylsarcosine or N-dimethylglycylglycine, this is particularly striking with the uterine extract, where no splitting was observed in 21 hours with 40 times the amount of enzyme necessary for complete hydrolysis of GG in 3 hours Although glycylsarcosine has previously been shown to be resistant to hydrolysis (5, 6), this has been reinvestigated because of the pronounced activation of GG dipeptidase by Co^{++}, which was unknown when the earlier work was performed

TABLE I

Specificity of Glycylglycine Dipeptidase

The experiments were performed at 40° in 0 1 M phosphate buffer at pH 7 6 to 7 7

Tissue	Substrate	Protein N per cc test solution	Time	Hydrolysis	
				0 001 M Co^{++}	No Co^{++}
		mg	*hrs*	*per cent*	*per cent*
Rat muscle	Glycylglycine	0 20	3	93	18
	Sarcosylglycine	0 60	20	63	13
	N-Dimethylglycylglycine	0 80	20	−2	3
	Glycylsarcosine	0 80	20	0	1
Human uterus	Glycylglycine	0 02	3	104	6
	Sarcosylglycine	0 40	3	102	3
	N-Dimethylglycylglycine	0 80	21	1	2
	Glycylsarcosine	0 80	21	2	−2

It has already been demonstrated that the hydrolysis of GG follows the kinetics of a zero order reaction with extracts of human uterus or rat muscle (1), this is likewise true for the enzyme of hog intestinal mucosa (Table II) With all three extracts, the hydrolysis of SG also follows the zero order kinetics K^0, the zero order velocity constant, is calculated as per cent hydrolysis per minute, and C^0 is K^0/E, where E is in mg of protein N per cc K^0_{SG} is proportional to the enzyme concentration, as shown in Table II for the uterine extract

The GG dipeptidase from hog intestinal mucosa is relatively labile even on standing in the cold After 5 days, C^0_{GG} decreased from 15 3 to 4 6, or 3 3 times Similarly, C^0_{SG} decreased from 1 07 to 0 29 or 3 7 times The parallel loss of activity for the two substrates is further evidence that the same enzyme is responsible for both actions Similar results have also been obtained with the labile GG dipeptidase of rat muscle

From the data in Table II, the proteolytic quotient Q, C^0_{GG}/C^0_{SG}, may be calculated for the three extracts For rat muscle Q = 11 7, for intestinal

TABLE II

Kinetics of Hydrolysis of Sarcosylglycine

The tests were made at 40° in the presence of 0 001 M $CoCl_2$ The solutions were buffered at pH 7 6 to 7 8 with 0 02 M veronal (rat muscle) or 0 1 M phosphate (uterus and intestinal mucosa) The preparation from intestinal mucosa was a fresh aqueous extract of an acetone dried powder, the aged extract had been allowed to stand at 5° for 5 days K^0 is the zero order velocity constant expressed as per cent hydrolysis per minute C^0, the zero order proteolytic coefficient, is K^0/E, where E is in mg of protein N per cc

Tissue	Substrate	Protein N per cc. test solution	Time	Hydrolysis	K^0	C^0
		mg	*min*	*per cent*		
Rat muscle	Glycylglycine	0 059			0 61	10 4
	Sarcosylglycine	0 108	180	17	0 094	
			240	23	0 096	
			300	29	0 097	0 89
Human uterus	Glycylglycine	0 041			0 69	16 8
		0 082			1 28	15 6
	Sarcosylglycine	0 082			0 31	3 8
		0 123			0 41	3 3
		0 205			0 62	3 0
		0 246			0 87	3 5
		0 41	15	19	1 27	
			30	38	1 27	
			45	56	1 24	
			60	75	1 25	3 1
Hog intestinal mucosa	Glycylglycine	0 0416	30	17	0 57	
			60	38	0 63	
			90	61	0 68	
			120	79	0 66	15 3
		0 0416	120	17*		
	Sarcosylglycine	0 208	90	19	0 21	
			150	36	0 24	
			180	40	0 22	
			300	65	0 22	1 07
			300	19*		
" " "	Glycylglycine	0 0416			0 19	4 6
(aged 5 days)	Sarcosylglycine	0 208			0 06	0 29

* These test solutions did not contain added Co^{++}

mucosa Q = 14 3, and for uterus Q = 4 8 If the activities were completely homospecific, Q should be the same for all three extracts With extracts

of rat muscle and hog mucosa, consistent values of Q were always obtained (Table III). However, with some extracts of human uterus values of 10 to 12 were found. While the data were reasonably consistent for C^0_{GG}, the variation appears to be in the rate of hydrolysis of SG. From this, it would appear that the GG dipeptidases of different tissues are homospecific, but there is probably some additional factor in uterine extracts which is concerned in the hydrolysis of SG.

TABLE III

Proteolytic Quotients for Glycylglycine Dipeptidases

The experiments were performed at 40° and pH 7.6 to 7.8 with 0.02 M veronal or 0.1 M phosphate in the presence of 0.001 M Co^{++}. The proteolytic quotient = C^0_{GG}/C^0_{SG}.

Preparation	Buffer	C^0_{GG}	C^0_{SG}	Proteolytic quotient
1 * Human uterus	Phosphate	16.2	3.4	4.8
2 " "	"	15.3	3.1	4.9
3 " "	"	18.8	4.6	4.1
4 " "	Veronal	13.8	1.37	10.1
5 " "	Phosphate	18.8	1.61	11.7
6 " "	Veronal	12.2	1.07	11.4
1 * Rat muscle	"	10.4	0.98	11.7
2 " "	"	10.3	0.81	12.7
3 " "	Phosphate	4.5	0.46	9.8
1 * Hog intestinal mucosa	"	15.3	1.07	14.3
2 " " "	"	14.5	0.89	16.3
3 " " "	"	11.5	1.03	11.2

* These are the preparations used for the experiments in Table II.

Hydrolysis of Sarcosyl-L-leucine

Thus far, the only other specific dipeptidase action has been ascribed to the enzymes of various tissues which split glycyl-L-leucine (GL) (2, 3). It was of interest to ascertain whether these enzymes are capable of acting on the analogous methylated peptide, sarcosyl-L-leucine (SL). It was found that the Mn^{++}-activated GL dipeptidase of hog intestinal mucosa does hydrolyze SL. The splitting of SL is also activated by Mn^{++}, and shows a first order rate of hydrolysis only after the enzyme has been preincubated with Mn^{++} (Table IV). Similarly, the Zn^{++}-activated dipeptidase of human uterus hydrolyzes SL and this hydrolysis is activated by Zn^{++} also. This splitting of SL likewise follows the kinetics of a first order reaction. It should be noted that for the intestinal preparation Q for C_{GL}/C_{SL} is 17, while for the uterine extract Q is 74. Obviously, the two systems do not appear to be completely homospecific.

Coordination Compounds of Cobalt and Peptides

Measurements of absorption spectra were made in a 1 cm quartz cell at room temperature with a Beckman ultraviolet spectrophotometer A solution of the compound was adjusted to pH 7 6 to 7 8 with 0 1 M NaOH, and $CoCl_2$ was added The final concentrations were 0 125 M for the compound and 0 01 M Co^{++} The spectrum was measured after the solution was allowed to stand for 18 to 24 hours Since no anaerobic precautions were taken, those compounds which coordinated with Co^{++} may have undergone some formation of the oxygen binding and oxidation complexes

TABLE IV

Hydrolysis of Sarcosyl-L-leucine

The tests were performed at 40° and pH 7 7 in 0 1 M phosphate The intestinal Preparation A was incubated at 40° for 3 hours with 0 02 M Mn^{++} before addition to the test solution C^1 is the first order proteolytic coefficient

Tissue	Substrate	Metal in test solution	Protein N per cc test solution	Time	Hydrolysis	C^1
			mg	*hrs*	*per cent*	
Hog intestinal mucosa	Glycyl-L-leucine	0 002 M Mn^{++}	0 103			0 031
	Sarcosyl-L-leucine	0 007 " "	0 362			0 0018
		None	1 03			0 00015
Human uterus	Glycyl-L leucine	0 001 M Zn^{++}	0 0197			0 31
	Sarcosyl-L-leucine	0 001 " "	0 59	0 5	16	0 0043
				1 0	31	0 0046
				1 5	40	0 0043
				2 0	46	0 0038
				2 5	56	0 0040
	"	None	0 59	1 0	18	0 0025
				2 0	30	0 0022
				2 5	35	0 0021

described by Burk *et al* (4) However, the interest in this study was whether coordination took place at all, and not in the secondary fate of the coordination compound in the absence of the enzyme It has already been demonstrated (1) that the product formed by the action of the enzyme on GG is glycine

The following glycine derivatives which are not hydrolyzed by GG dipeptidase showed no change or only a very small increase in the intensity of the Co^{++} absorption spectrum glycinamide, benzoylglycine, benzoylglycylglycine, carbobenzoxyglycylglycine, glycylsarcosine, and N-dimethylglycylglycine The sparingly soluble benzoylglycinamide and carbobenzoxyglycylglycinamide did not show any reaction with Co^{++} In

addition, no coordination took place with β-alanylglycine or β-alanyl-β-alanine, but glycyl-β-alanine and glycyl-L-alanine did show slight evidence of coordination

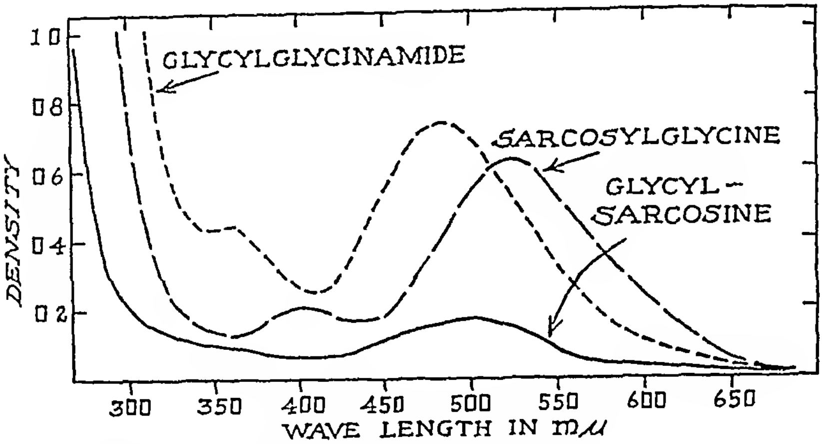


FIG 1 Absorption spectra of sarcosylglycine, glycylsarcosine, and glycylglycinamide after standing with 0 01 M $CoCl_2$ for 24 hours

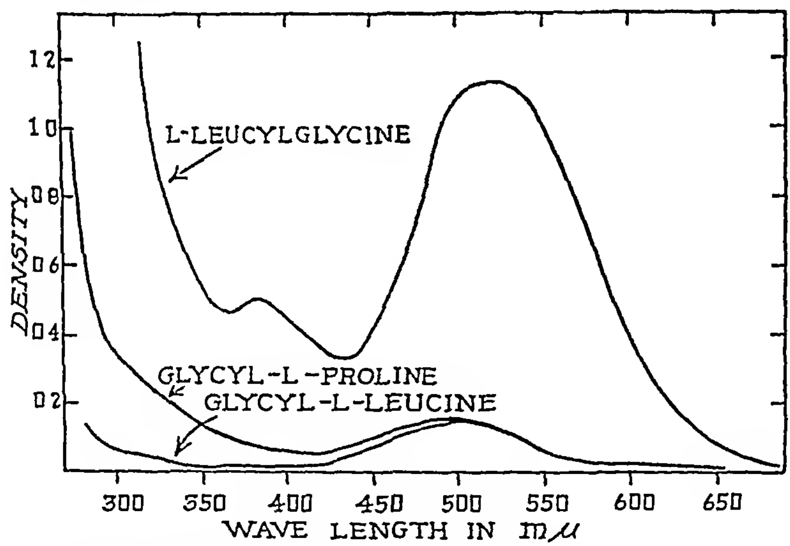


FIG 2 Absorption spectra of L-leucylglycine, glycyl-L-leucine, and glycyl-L-proline after standing with 0 01 M $CoCl_2$ for 24 hours

It has already been reported (1) that GG forms a compound which gives a strong intensification of the absorption bands of Co^{++} This compound has its maximal absorption of 520 mμ Sarcosylglycine forms a similar complex, and the maximal absorption in the visible is at the same wavelength (Fig 1) However, the intensity of the band is somewhat less than that of the GG complex Glycylglycinamide, which is resistant to the dipeptidase, also forms a coordination complex with Co^{++} (Fig 1) How-

ever, while the height of the band is like that of SG rather than GG, the position of the maximum is at 480 mμ The spectrum of the weak complex of Co^{++} with glycylsarcosine is also shown in Fig 1 $CoCl_2$ (0 01 M) has an absorption maximum at 510 mμ with an optical density of 0 05

It was found that L-leucylglycine coordinates much more strongly with Co^{++} than does glycyl-L-leucine (Fig 2) Obviously, there is considerable specificity in the formation of these complexes, even with unsubstituted peptides The peptide glycyl-L-proline, which does not possess a peptide hydrogen, shows only weak coordination with Co^{++}

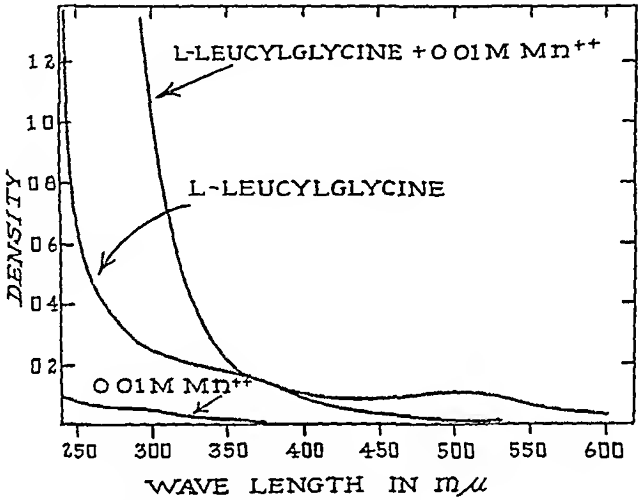


FIG 3 The absorption spectrum of L leucylglycine (0 125 M), which had been allowed to stand with 0 01 M $MnCl_2$ for 24 hours at room temperature This is compared with the spectrum of the free peptide and 0 01 M $MnCl_2$

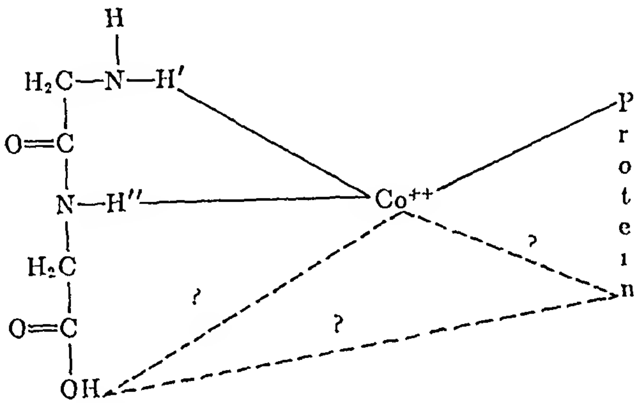
Complex formation also occurs with other metals and peptides Fig 3 shows the change in the absorption spectrum which occurs with L-leucylglycine and Mn^{++} at pH 7 7 A similar but weaker increase in absorption may also be demonstrated with L-leucylglycine and Mg^{++} This is of considerable interest, since the ubiquitous leucine aminopeptidase which splits this peptide is activated by Mn^{++} and Mg^{++}

DISCUSSION

An attempt has been made to test the hypothesis that the rôle of metals in hydrolytic and other non-oxidative reactions is to act as a bridge in the formation of the enzyme-substrate complex This has been investigated by the study of complex formation between Co^{++} and a series of compounds related to glycylglycine, since this peptide is hydrolyzed by a specific enzyme which requires Co^{++} for its activity An obvious parallelism has

been found between the compounds which form a Co^{++} complex and those which are split by the dipeptidase This is summarized in Table V The data are taken from this paper and an earlier one (1)

The data indicate to some extent the manner in which complex formation must take place [1] Since sarcosylglycine forms a compound with Co^{++} and



is hydrolyzed by the dipeptidase, one free hydrogen (H′) is sufficient on the amino group The nitrogen must be basic since the acylated compounds, carbobenzoxyglycylglycine and benzoylglycylglycine, do not coordinate and are not split The inactivity of the strongly basic compound N-dimethylglycylglycine shows that basicity is not a sufficient requirement, one free hydrogen must be present on the terminal amino group

It is well known that Co^{++} coordinates best with compounds like ethylenediamine, where chelate rings are possible Therefore, the peptide hydrogen (H″) must be present for a chelate complex to be formed The weak complex of glycylsarcosine with Co^{++} shows this to be the case, and this compound is completely resistant to the enzyme The tendency of Co^{++} to form five-membered chelate rings is illustrated by the failure to observe any coordination with β-alanylglycine and β-alanyl-β-alanine, neither of these compounds is hydrolyzed by GG dipeptidase (7) However, the enzyme may have a slow action on glycyl-β-alanine (7) and there is some evidence of weak coordination of this compound with Co^{++}

Glycylglycinamide is not hydrolyzed by the dipeptidase However, it does form a chelate complex with Co^{++} but of a somewhat different character than glycylglycine as indicated by the shift of 40 mμ in the principal absorption band Whether the free carboxyl group is necessary for com-

[1] In the absence of the enzyme, coordination probably involves 2 moles of the compound with 1 of Co^{++} for those substances in which formation of the chelate compound is possible However, with molecules like glycine, 4 moles would coordinate with each Co^{++}

bination with Co^{++} or with the protein is still undecided, although it would seem probable that Co^{++} forms a chelate complex with the protein also Of the four coordination places which Co^{++} has available, two must be with the peptide, and at least one with the protein Complex formation between metals and proteins is, of course, quite well known (8) The fact that for certain enzymes the activation by the metal is a slow reaction indicates that complex formation, and not a simple ionic reaction, is occurring This was first described for leucine aminopeptidase (9, 10)

TABLE V

Action of Glycylglycine Dipeptidase and Complex Formation with Co^{++}

The optical densities (1 cm cell) were measured with a solution of the compound (0 125 M) and $CoCl_2$ (0 01 M) at pH 7 6 to 7 8 after 18 to 24 hours at room temperature

Compound	Hydrolysis by dipeptidase	Optical density	Wave length
			$m\mu$
Glycylglycine	++++	2 0*	520
Sarcosylglycine	++	0 60	520
N-Dimethylglycylglycine	−	†	
Glycylsarcosine	−	0 17	500
Glycylglycinamide	−	0 73	480
Glycinamide	−	0 15	500
Glycine		0 16	500
Triglycine	−	0 49	500
Benzoylglycylglycine	−	0 06	510
β-Alanylglycine	−	†	
β-Alanyl β alanine	−	†	
Glycyl-β alanine	±	0 17	500
Glycyl L alanine	−	0 28	505
0 01 M $CoCl_2$		0 06	510

* After 3 hours, the value was 0 52

† Precipitation of gelatinous cobalt hydroxide

and has since been found for arginase (11), phosphatase (12), prolidase (13), and glycyl-L-leucine dipeptidase (3) The rate of such reactions seems to vary quite widely, and to depend on many factors The more highly purified enzymes seem to react more slowly than do many crude preparations Thus far, we have been unable to detect any activation time of glycylglycine dipeptidase and Co^{++}, nor have we observed any increase in rate of splitting of GG by prior incubation of substrate and Co^{++}

It is apparent from this hypothesis that a part of the specificity of the enzyme must reside in the ability of the metal to undergo complex formation both with the substrate and with the protein This would help to

explain the extreme metal specificity shown by many enzymes, including the peptidases However, this must be stated as a necessary condition for enzyme action, but is not a sufficient condition, since the major enzyme specificity must be due to the protein moiety

Green, Herbert, and Subrahmanyan (14) have proposed that the rôle of the metal ion in carboxylase is to bind diphosphothiamine to the protein moiety of the enzyme However, their data do not exclude the possibility that the linkage is in the order protein-diphosphothiamine-metal With the peptidases, no other dissociable prosthetic group other than metal has yet been found, although leucine aminopeptidase and prolidase have now been extensively purified Here the metal must form the link between the enzyme and the substrate

Thus far, the hypothesis of the rôle of Co^{++} in the action of glycylglycine dipeptidase has fulfilled every test to which it has been subjected It is likely that this proposal can be extended to other peptidases and additional types of enzymes No other suggestion to explain why metals are required in hydrolytic reactions has been proposed, and it is difficult to conceive of any other function which these metals could have

The assistance of Douglas M Brown and Mrs Toshiko Shimizu is gratefully acknowledged

EXPERIMENTAL

Hydrolysis was measured on 0 2 cc samples by the liberation of carboxyl groups by the method of Grassmann and Heyde (15) The substrates were used at 0 05 M in 2 5 cc volumetric flasks The rat muscle extract and the acetone-dried preparation of human uterus have already been described (1) For glycylglycine dipeptidase, a fresh aqueous extract of hog intestinal mucosa was used For glycyl-L-leucine dipeptidase, the crude aqueous extract of mucosa was precipitated with ammonium sulfate and dialyzed (3)

Sarcosyl-L-leucine—6 gm of chloroacetyl-L-leucine (16) were allowed to stand in a pressure bottle at room temperature for 2 days with 30 cc of 35 per cent aqueous methylamine The solution was diluted with water and concentrated to a thick syrup under reduced pressure repeatedly with ethanol The gelatinous residue was dissolved in ethanol and precipitated with ether Yield, 5 1 gm The compound was recrystallized from ethanol as needles, m p 187°

$C_9H_{18}O_3N_2$	Calculated	C 53 4, H 9 0, N 13 9
202 3	Found	" 53 4, " 9 0, " 13 8

$$[\alpha]_D^{21.6} = -30.4° \text{ (2.2\% in water)}$$

N-Dimethylglycylglycine Hydrochloride—5 gm of chloroacetylglycine (17) were dissolved in 20 cc of 25 per cent dimethylamine and allowed to stand at room temperature for 4 days The solution was concentrated *in vacuo* repeatedly with ethanol The thick syrup was taken up in hot butanol and filtered On cooling the solution, 3 4 gm of the crystalline product were obtained In order to remove the dimethylamine hydrochloride, the substance was dissolved in water and crystallized as fine needles by the addition of ethanol After one more recrystallization, the melting point was 186–187°

$C_6H_{13}O_3N_2Cl$	Calculated	C 36 7, H 6 7, N 14 3
196 6	Found	" 36 7 " 6 6, " 14 3

Sarcosylglycine—This was prepared as described by Levene, Simms, and Pfaltz (17)

Glycylsarcosine—This was synthesized by the procedure of Bergmann, Zervas, Schleich, and Leinert (6)

SUMMARY

1 Preparations of glycylglycine dipeptidase from human uterus, rat muscle, and hog intestinal mucosa hydrolyze sarcosylglycine This hydrolysis, like that of glycylglycine, is strongly activated by Co^{++}, and follows the kinetics of a zero order reaction The enzyme has no demonstrable action on glycylsarcosine or N-dimethylglycylglycine

2 The glycyl-L-leucine dipeptidases of hog intestinal mucosa (Mn^{++} activation) and human uterus (Zn^{++} activation) hydrolyze sarcosyl-L-leucine These splittings follow first order reaction kinetics

3 Co^{++} coordinates readily with glycylglycine but not with most of the substituted derivatives of the dipeptide In view of the parallelism between the ability of Co^{++} to coordinate and the ability of the enzyme to act, it is proposed that the rôle of metals in hydrolytic reactions is to act as a bridge in the formation of the enzyme-substrate complex

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THE SYNTHESIS OF S-BENZYLTHIOPYRUVIC ACID AND ITS CONVERSION TO N-ACETYL-S-BENZYL-L-CYSTEINE IN THE RAT, UNAVAILABILITY OF THIOPYRUVIC ACID TO RATS FOR GROWTH PURPOSES*

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Some time ago we demonstrated the acetylation of S-benzyl-L-cysteine in the dog, rabbit, rat, and man (1–3) Administration of S-benzyl-D-cysteine to rats (4, 5) or man (3) was followed by the excretion of some N-acetyl-S-benzyl-L-cysteine, thus indicating a partial inversion of the D derivative of cysteine to the optical antipode Ample experimental evidence is available to show that oxidative deamination of D-amino acids occurs *in vivo* and *in vitro* In an attempt to explain the inversion of S-benzyl-D-cysteine *in vivo*, it has been postulated that S-benzylthiopyruvic acid is formed *in vivo* as the intermediate (2–5) S-Benzyl-L-cysteine is readily deaminated oxidatively by rat kidney preparations (6), and a hydrazone of a keto acid has been isolated from rabbit urine following the administration of S-benzyl-L-cysteine, which was presumably the hydrazone of S-benzylthiopyruvic acid (7) Previous experiments have shown that the α-hydroxy analogues of S-benzyl-L- and S-benzyl-DL-cysteine are excreted unchanged by the rat, suggesting that the rat is unable to oxidize the hydroxy acid or to aminate it (8)

In the present study we prepared S-benzylthiopyruvic acid and administered it to adult rats From the urine of these animals we isolated N-acetyl-S-benzyl-L-cysteine and identified it in a conventional manner The demonstration of the asymmetric synthesis of L-cysteine derivative from the corresponding keto acid raised the question of a similar synthesis of L-cysteine from thiopyruvic acid *in vivo* It will be recalled from earlier studies that D-cystine could not replace L-cystine in the diet of the rat for growth purposes (9), although D-cystine was shown to undergo ready oxidative deamination *in vitro* by rat kidney preparations (10) It thus appeared possible that D-cystine undergoes oxidative deamination *in vivo* also, but the resulting thiopyruvic acid is metabolized before the synthesis of L-cystine can occur It should be pointed out, however, that in studies on

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rabbits (11) and dogs (12) it was found that the sulfur of D-cystine is less readily oxidized than that of the L isomer In order to test the availability of thiopyruvic acid for growth purposes, we synthesized the acid and administered it to growing rats orally or subcutaneously No growth stimulation was observed, although under similar conditions L-cystine gave the usual growth response Since thiopyruvic acid is readily oxidized *in vivo* to yield inorganic sulfate in the urine (13), these data seem to suggest that thiopyruvic acid, particularly the thiol group, is metabolized before the animal organism can utilize it for L-cysteine synthesis Once the thiol group of thiopyruvic acid is stabilized by the benzyl group (as in S-benzylthiopyruvic acid), the synthesis of the L-cysteine derivative can and does take place [1]

In discussions on N,N′-dimethylcystine (14) the argument was advanced that demonstration of the growth-promoting power of the methylated amino acid was tantamount to demonstrating that its keto analogue was also capable of supporting growth The data presented here seem to suggest that the growth-promoting property of N,N′-dimethyl-L-cystine cannot definitely be ascribed to the formation of the keto analogue *in vivo* Hydrolysis of the methyl groups *in vivo* to yield L-cystine appears the more likely possibility Had the keto analogue been formed from N,N′-dimethyl-L-cystine, no growth would have resulted from its formation It must be admitted, however, that it cannot be assumed *a priori* that the administration of thiopyruvic acid to the rat is equivalent to the situation when such a keto acid is formed in the tissues

The method which we used for the synthesis of S-benzylthiopyruvic acid is based on that reported by Parrod (15) We found, however, that considerable simplification can be introduced in the procedure, particularly in the steps involving the use of sulfuryl chloride It was also found that pyruvic acid can be directly chlorinated with sulfuryl chloride to yield chloropyruvic acid A brief description of the methods of preparation of chloropyruvic and S-benzylthiopyruvic acids and their ethyl esters, and of sodium thiopyruvate, is presented As far as we are aware, S-benzylthiopyruvic acid has not been previously prepared

EXPERIMENTAL

Pyruvic Acid Ethyl Ester—33 2 gm of pyruvic acid dissolved in 100 ml of absolute ethanol which contained 0 3 ml of concentrated sulfuric acid were refluxed for 3 hours Excess ethanol was distilled off at atmospheric

[1] In a private communication Dr J Parrod has just informed us that he isolated *p*-iodophenylmercapturic acid from the urine of rabbits which were fed S-*p*-iodophenylthiopyruvic acid The synthesis of a cysteine derivative from a corresponding keto acid is thus not apparently confined to the organism of the rat

pressure on a water bath The ester was collected, boiling at 44–45° at 10 mm The yield was 50 to 60 per cent

Chloropyruvic Acid Ethyl Ester—60 gm of pyruvic acid ethyl ester were dissolved in 220 ml of sulfuryl chloride and the solution was heated on a water bath at 60–65° for 1 5 hours The temperature was then gradually raised to 90–95° during the next 1 5 hours Excess sulfuryl chloride was removed by distillation at atmospheric pressure Chloropyruvic acid ethyl ester was collected at 79° at 9 mm Parrod (15) reported that chloropyruvic acid ethyl ester boils at 90° at 15 mm The yield was 60 to 70 per cent

Chloropyruvic Acid—The compound was obtained by direct chlorination of pyruvic acid with sulfuryl chloride, as was described above Chloropyruvic acid was collected, of boiling point 90–97° at 8 to 13 mm The compound solidifies in a cool condenser and it is necessary to warm the condenser for efficient collection of the compound Colorless shiny soft crystals were obtained The compound is a strong vesicant The yield was 50 to 65 per cent For analysis it was dried at room temperature *in vacuo* over P_2O_5

$C_3H_3O_3Cl$	Calculated	C 29 50, H 2 46, Cl 31 15
	Found	" 29 06, " 2 55, " 31 60

S-Benzylthiopyruvic Acid Ethyl Ester—12 4 gm of benzyl mercaptan were added to 100 ml of water containing 5 6 gm of KOH With vigorous shaking of the solution, 15 1 gm of chloropyruvic acid ethyl ester were added in several portions, the reaction mixture being kept alkaline (not below pH 9 to 10) by addition of extra pellets of KOH The reaction vessel was kept immersed in an ice bath After all of the chloropyruvic acid ethyl ester had been added, the flask was shaken for 15 to 20 minutes, allowed to stand at room temperature for 1 hour, then placed in the refrigerator for 3 hours The mass which separated on standing was removed by decantation of the supernatant fluid and washed with cold water The semisolid mass was then extracted with ether The ether extract was washed with water, then dried with sodium sulfate The ether was removed by distillation, and the residue was subjected once more to ether-water purification The purified material was dried *in vacuo* at room temperature over P_2O_5 The compound remained a heavy oil after standing for several days at 2°

$C_{12}H_{14}O_3S$	Calculated	C 60 50, H 5 88, S 13 44
	Found	" 60 10, " 6 10, " 13 63

2,4-Dinitrophenylhydrazone of S-Benzylthiopyruvic Acid Ethyl Ester—0 2 gm of 2,4-dinitrophenylhydrazine was dissolved in a little concentrated sulfuric acid and the solution was diluted with 9 volumes of absolute

ethanol To the solution 0 23 gm of S-benzylthiopyruvic acid ethyl ester was added, and the mixture was gently warmed On cooling, the crystals of the hydrazone separated which were collected and recrystallized three times from absolute ethanol After being dried at 100° *in vacuo* over P_2O_5, the compound melted at 138–139° (uncorrected)

$C_{18}H_{18}N_4O_6S$	Calculated	C 51 67, H 4 31, N 13 39, S 7 66
	Found	" 51 96, " 4 50, " 13 50, " 7 96

S-Benzylthiopyruvic Acid—To a solution of 13 2 gm of benzyl mercaptan in 100 ml of water containing about 8 gm of KOH, 13 gm of chloropyruvic acid were added in the manner described above Upon completion of the reaction, the solution was extracted with ether, and the aqueous layer was acidified with HCl to pH 1 or 2 A semisolid mass separated almost immediately It was removed by filtration, washed with cold water, then extracted with ether The ether extract was dried with sodium sulfate, and the ether removed by distillation The residue was further purified by extraction with ether, washing with water, drying as before, and distillation of the ether The residue solidified at room temperature in a desiccator over sulfuric acid without formation of crystals

$C_{10}H_{10}O_3S$	Calculated	C 57 14, H 4 76, S 15 24
	Found	" 57 69, " 5 00, " 15 55

2,4-Dinitrophenylhydrazone of S-benzylthiopyruvic acid was prepared as described above After drying *in vacuo* at 100° over P_2O_5, the compound melted at 148–149° (uncorrected)

$C_{16}H_{14}O_6N_4S$	Calculated	C 49 70, H 3 86, N 13 95, S 8 52
	Found	" 49 23, " 3 60, " 14 30, " 8 20

Sodium Thiopyruvate—The compound was prepared essentially as was described by Parrod (15) On recrystallization from water by addition of ethanol the compound separated in the form of beautiful needles which contained 2 molecules of water of crystallization

$NaC_3H_3O_3S\ 2H_2O$	Calculated	C 20 23, H 3 93, S 17 92
	Found	" 20 40, " 4 25, " 17 86

Feeding Experiments

A complete 25 per cent casein diet which contained 1 0 per cent of S-benzylthiopyruvic acid was fed to four adult male rats of the Wistar strain The rats had not previously been used for experiment The urine was collected daily over a period of 5 days and preserved in a refrigerator About 150 gm of the food were consumed by the rats during this period The pooled urine was then extracted in the manner described previously for the isolation of N-acetyl-S-benzyl-L-cysteine (1–3) The yield of the

product obtained averaged 150 mg per gm of the keto acid ingested In addition to N-acetyl-S-benzyl-L-cysteine, some unchanged S-benzylthiopyruvic acid was excreted by the rats This was identified as the 2,4-dinitrophenylhydrazone, m p 148–149° After drying *in vacuo* at 100° over P_2O_5, the isolated N-acetyl-S-benzyl-L-cysteine melted at 143–144°, and the melting point remained unchanged when the compound was mixed with an authentic sample of N-acetyl-S-benzyl-L-cysteine

$C_{12}H_{15}O_3NS$	Calculated	C 56 92, H 5 93, N 5 54, S 12 65, Acetyl 16 98
	Found	" 57 16, " 6 20, " 5 28, " 12 43, " 16 38

The isolated product had the specific rotation of $[\alpha]_D^{23} = -44°$ for a 1 per cent solution in ethanol

S-Benzyl-L-cysteine was prepared from the isolated material by hydrolysis of the compound in dilute HCl in the usual manner and by precipitation of the cysteine derivative with ammonia On recrystallization from water, S-benzyl-L-cysteine melted at 212–213° and had the specific rotation of $[\alpha]_D^{23} = +23°$ for a 1 per cent solution in N NaOH

Growth Experiments

Three litters of albino male rats of Wistar strain were used They were divided into three groups, six animals in each group One group was fed an 8 per cent casein diet, the second the same diet supplemented with 1 per cent of sodium thiopyruvate, and the third group received 0 5 per cent of L-cystine as the supplement to the casein diet The exact composition of the diet was described previously (16) After 6 weeks on these diets, the control group and the one ingesting thiopyruvic acid gained on the average of 1 2 gm per day per rat, while the third group ingesting L-cystine gained on the average of 2 9 gm per day per rat Similar results were obtained when 75 mg of sodium thiopyruvate in water were injected subcutaneously per rat per day in two equal portions 4 hours apart It is thus evident that sodium thiopyruvate does not stimulate the growth of rats when administered either orally or subcutaneously under the conditions of our experiments

SUMMARY

1 S-Benzylthiopyruvic acid was synthesized and fed to adult rats From the urine of these animals N-acetyl-S-benzyl-L-cysteine was isolated and identified

2 Sodium thiopyruvate does not stimulate the growth of rats when administered orally or subcutaneously in lieu of L-cystine

3 It is concluded that the rat is able asymmetrically to synthesize S-benzyl-L-cysteine from the corresponding keto analogue Since sodium

thiopyruvate did not stimulate the growth of rats in lieu of L-cystine, it appears that the keto acid is metabolized prior to its conversion to L-cysteine or L-cystine.

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THE EFFECT OF RELATED IONS ON THE POTASSIUM REQUIREMENT OF LACTIC ACID BACTERIA*

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The essential nature of K^+ for growth of lactic acid bacteria has previously been established (1) The amount required for maximum growth seemed unusually high Further investigation showed that the quantitative requirement for potassium was markedly influenced by the amounts of sodium and ammonium ions in the medium These findings prompted an extension of the investigation to other alkali metal ions The results of these studies, which provide examples of "ion antagonism" among the alkali metals and which are most readily interpreted as due to competitive inhibition of the essential rôle of potassium by related ions, are presented below

EXPERIMENTAL

Cultures and Inocula—*Lactobacillus arabinosus* 8014, *L casei* 7469, *Leuconostoc mesenteroides* 8042 and 9135, and *Streptococcus faecalis* 8043 were carried as stab cultures in yeast-dextrose agar Inoculum cultures were incubated 16 to 18 hours in a previously described medium (1) The cultures of *L mesenteroides* 9135 were incubated at 30°, the remainder at 37°

Basal Medium—To study the effect of the alkali metal and ammonium ions on the growth of the organisms, a medium as free as possible of these ions was prepared A sulfuric acid hydrolysate of casein was used as the nitrogen source in the medium, the sulfate ion being removed with barium hydroxide (2) For rapid and heavy growth, acetate and phosphate are required These are ordinarily employed as sodium or potassium salts, which was not desirable in this instance To permit addition of these and other anions to the medium, preliminary experiments were run to determine the suitability of various amines as the base for neutralization of the medium Of several tried, triethanolamine was found most satisfactory, since it was highly soluble and was non-toxic for the various test organisms at the concentrations required The composition of the basal medium is given in Table I The pH of the medium was adjusted to 6 8 with a 10 per cent

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aqueous solution of triethanolamine The amounts of Mn^{++} and Mg^{++} are considerably higher than those normally used in the presence of an acetate buffer (1) These amounts are required for rapid growth in the presence of triethanolamine, perhaps because triethanolamine, like citrate (1), forms complexes with these cations, thus lowering their effective concentrations

TABLE I

Composition of Basal Medium

	Amount per 10 cc final medium
	mg
Acid-hydrolyzed casein*	100
Asparagine	1
DL-Tryptophan	0 5
Cystine	1
Adenine HCl	0 1
Guanine HCl	0 1
Uracil	0 1
Glucose	100
Acetic acid†	45
Phosphoric acid†	3
$MgSO_4$ $7H_2O$	10
	γ
$MnSO_4$ H_2O	600
Pyridoxal HCl	1
Thiamine	1
Calcium pantothenate	2
Riboflavin	2
Niacin	2
p-Aminobenzoic acid	1
Folic acid	0 05
Biotin	0 01

* See the text

† The required amounts of acetic and phosphoric acids were neutralized with triethanolamine (Eastman Kodak, Practical) before addition to the medium The same base was used to adjust the initial pH of the medium to 6 8

This medium supported little or no growth of the organisms tested unless K^+ was added Preliminary pretreatment to remove traces of K^+ (1) was thus omitted

Test Procedure—The testing procedures were those customarily used in microbiological assay work and have been previously described (1) Unless otherwise specified, cultures were incubated for 24 hours, and metallic ions were added as the following salts K^+, K_2SO_4, Na^+, Na_2SO_4, NH_4^+, $(NH_4)_2SO_4$, Rb^+, RbCl, Cs^+, CsCl, and Li^+, LiCl

Sodium-Potassium Relationship—The effect of increasing levels of Na^+ on the growth response of *Lactobacillus casei* to K^+ is shown in Fig 1 Sodium ion inhibits growth of this organism in the presence of low concentrations of K^+ The inhibitory action of Na^+ is completely reversed if sufficient K^+ is added As the level of Na^+ is increased, the amount of K^+ required to prevent the resulting inhibition is also increased Four other lactic acid bacteria showed similar behavior The antibacterial indices for half maximum inhibition, *i e* the molar ratio of Na^+ to K^+ at which half

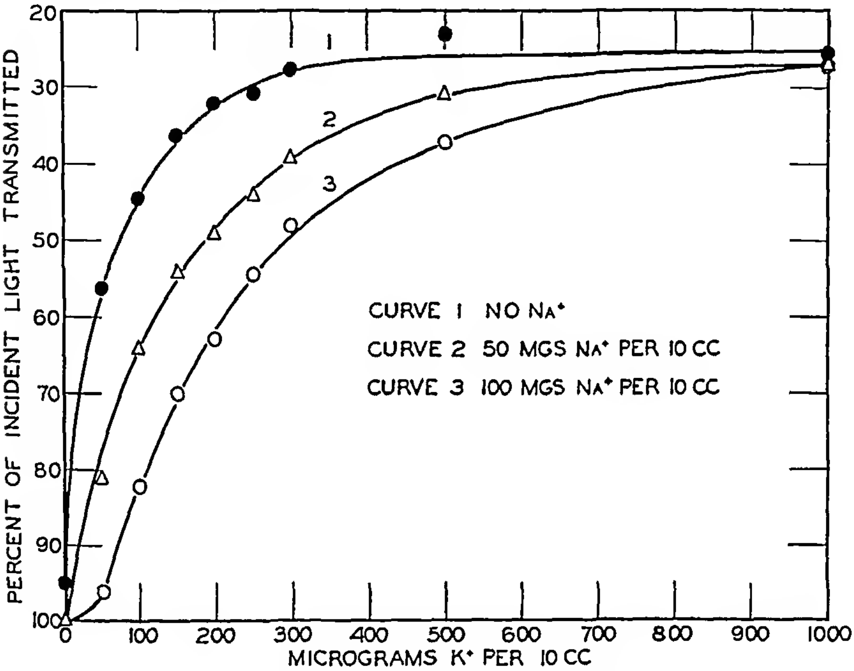

FIG 1 Effect of Na^+ on response of *Lactobacillus casei* to K^+

maximum growth of each organism was obtained, are presented in Table II for several different concentrations of Na^+ Although the inhibitory effects of Na^+ are overcome in each case by additional quantities of K^+, the sensitivity of various organisms to the inhibitor (Na^+) varies considerably The antibacterial indices are not entirely constant, but tend to increase with increasing concentration of Na^+ At the higher concentrations of Na^+ these ratios approach a constant value The reasons for this variation are not clear, its magnitude, however, is similar to that frequently observed with organic metabolite-antimetabolite combinations (3) For *Leuconostoc mesenteroides* 8042 the inhibitory action of Na^+ at the concentrations tested is not due entirely to an uncomplicated antagonism involv-

ing K^+ The addition of large amounts of K^+ only partially reverses the inhibitory action of Na^+ for this organism The nature of the inhibition not reversed by K^+ has not been investigated

The same relation between Na^+ and K^+ concentrations apparent from Fig 1, which presents data obtained following 24 hours of incubation, was found in separate trials after 120 hours incubation The inhibitory effect of Na^+ is thus apparently not due solely to retardation of the rate of growth.

The highest concentration of Na^+ used, *i e* 100 mg, corresponds to approximately a 3 per cent solution of sodium sulfate The question arose as to what extent the inhibition by Na^+ was due to the non-specific effects of high salt concentration (osmotic pressure effect, etc) or to the effect of the anion associated with the salts Fig 2 shows the growth response of

TABLE II

Molar Ratios of Na^+ to K^+ Which Permit Half Maximum Growth of Lactic Acid Bacteria at Various Levels of Added Na^+

Organism	Mg Na^+ per 10 cc medium			
	25	50	75	100
	Antibacterial index*			
Lactobacillus arabinosus	472	620	725	765
" *casei*	530	640	877	895
Streptococcus faecalis	340	530	688	805
Leuconostoc mesenteroides 8042	173	268	350	322
" " 9135		1890	2120	1990

* The antibacterial index is the molar ratio of inhibitor to metabolite (in this case Na^+ to K^+) at which growth is reduced to one-half of the maximum

Lactobacillus arabinosus to very high concentrations of K^+, added as KCl and K_2SO_4 Under our conditions neither salt is significantly inhibitory below a concentration of 200 mg of K^+ per 10 cc Above this concentration, the anion determines to a considerable extent the degree of inhibition Plotted in this way, the toxicity of the sulfate ion appears much less than that of the chloride ion This, however, is true because only half the molar concentration of $SO_4^=$ as of Cl^- is required to furnish the same amount of K^+ On a molar basis $SO_4^=$ and Cl^- are about equally toxic

These experiments indicate that, since Na^+ was added as the sulfate and at concentrations not exceeding 100 mg per 10 cc, the observed inhibition of *Lactobacillus arabinosus* was not to any appreciable extent due either to high salt concentration or to the presence of the anion At concentrations considerably higher than those used here, sodium salts will, of course, become toxic due to osmotic and perhaps other effects, and such toxicity will

not be alleviated by additions of K^+ At the concentrations used in this investigation, however, the principal toxic action of Na^+ appears to be due to its interference with K^+ utilization, and is prevented competitively by additions of K^+

These results prompted investigation of the possible relationship of other alkali metal ions and of NH_4^+ to the requirement for K^+ These results are summarized below

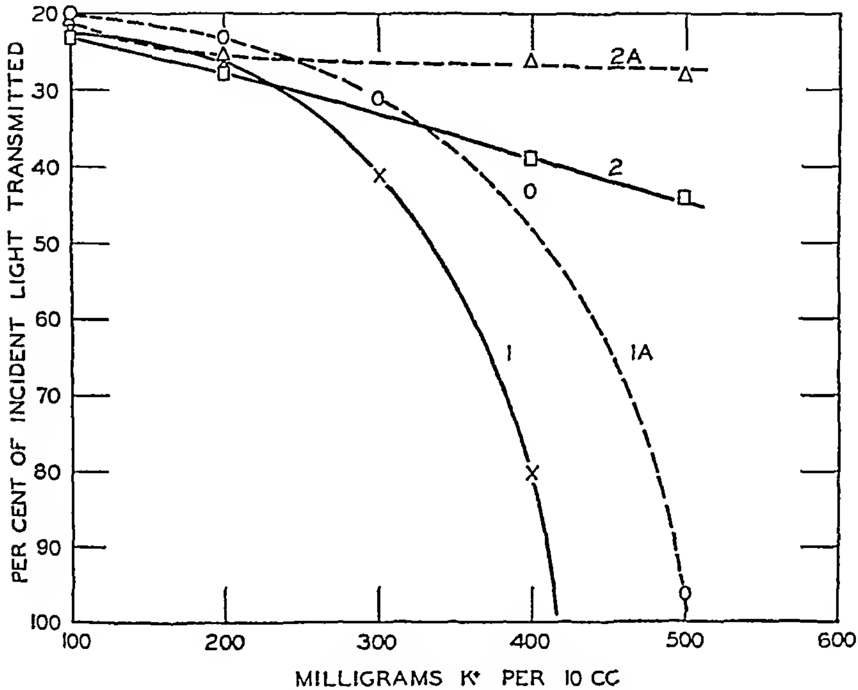

FIG 2 Inhibitory effects of high concentrations of potassium salts on growth o *Lactobacillus arabinosus* Curve 1, KCl, 24 hours incubation, Curve 1A, KCl, 48 hours incubation, Curve 2, K_2SO_4, 24 hours incubation, Curve 2A, K_2SO_4, 48 hours incubation

Ammonium-Potassium Relationship—The effect of NH_4^+ on the response of five lactic acid bacteria to K^+ was determined The results obtained with *Lactobacillus casei* are presented in Fig 3 The ammonium ion, like Na^+, is inhibitory to growth, and the inhibition is alleviated by increasing amounts of K^+ The concentration of K^+ required to reverse the inhibition increases as the amount of NH_4^+ increases A similar relationship was observed with the other four organisms tested The ratios of the concentration of NH_4^+ to K^+, at which half maximum inhibition of growth of

the five lactic acid bacteria occurs, are shown in Table III As with Na^+, the lowest concentration of NH_4^+ gives a lower value for this ratio than do higher levels The remaining ratios are much more nearly constant for each organism than are those for Na^+, and indicate the competitive nature of the inhibition The values obtained with *Leuconostoc mesenteroides* 8042 show the greatest variation, though for this organism, too, the toxicity of NH_4^+ is almost completely overcome by K^+ Comparison of the antibacterial indices for NH_4^+ (Table III) and Na^+ (Table II) shows that NH_4^+

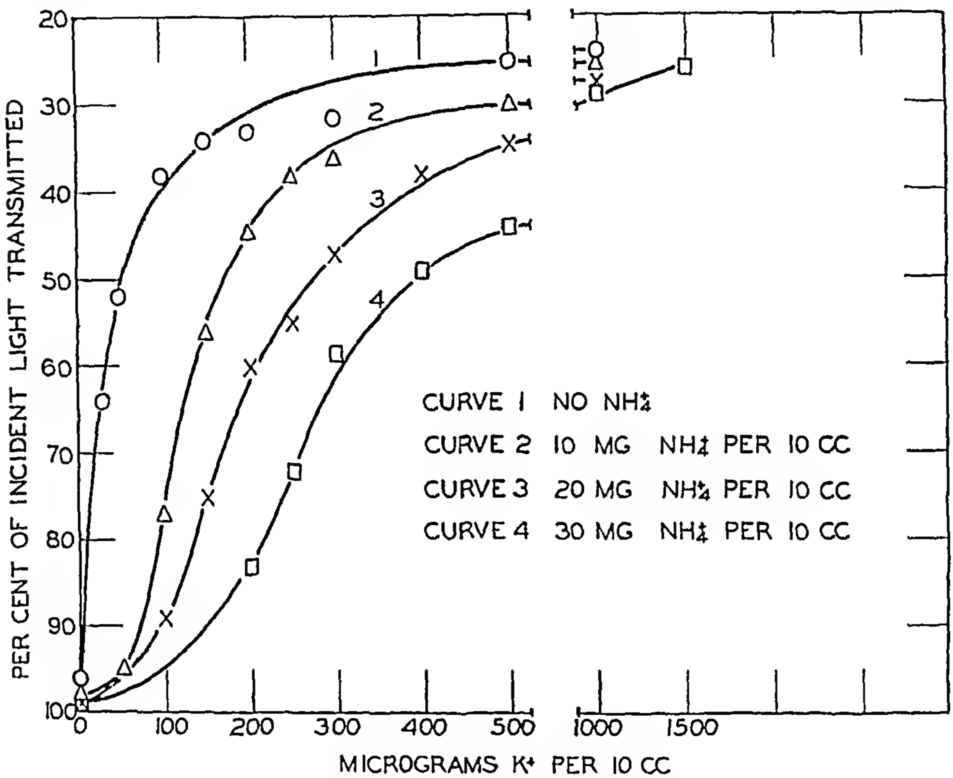

FIG 3 Effect of NH_4^+ on response of *Lactobacillus casei* to K^+

is considerably more effective than Na^+ in counteracting the growth-promoting effects of K^+

To determine whether the relationship observed above for lactic acid bacteria holds more generally, the effect of Na^+ and NH_4^+ on the response of the yeast, *Saccharomyces carlsbergensis* 4228, to K^+ was determined The medium (Table I) was supplemented with Ca^{++} (1 mg per 10 cc), inositol (250 γ per 10 cc), Fe^{++} (10 γ per 10 cc), and the pH adjusted to 5 5 to support more favorably the growth of this organism Cultures were shaken mechanically at 30° for 24 hours From Table IV it is evident that both ions interfere with the utilization of K^+ by the yeast in a manner analogous to their effect in lactic acid bacteria In contrast to the response of the

bacteria, however, the inhibitory effect of Na^+ is greater than that of NH_4^+ for yeast The yeast is considerably more sensitive to Na^+ than are the lactic acid bacteria tested

These findings permit a partial explanation for the high potassium requirement previously noted (1) for lactic acid bacteria This requirement was determined in a medium which contained considerable amounts of

TABLE III

Molar Ratios of NH_4^+ to K^+ Permitting Half Maximum Growth at Various Levels of Added NH_4^+

Organism	Mg NH_4^+ per 10 cc. medium			
	10	20	30	40
	Antibacterial index*			
Lactobacillus arabinosus	115	135	149	140
" *casei*	176	238	238	230
Streptococcus faecalis	88	105	112	115
Leuconostoc mesenteroides 8042	79	144	178	204
" " 9135	230	268	280	208

* As in Table II

TABLE IV

Effect of Na^+ and NH_4^+ on Response of Saccharomyces carlsbergensis 4228 to K^+

K^+ per 10 cc	Mg Na^+ per 10 cc				Mg NH_4^+ per 10 cc		
	0	25	50	75	25	50	75
	Per cent of incident light transmitted*						
γ							
0	93	100	100	100	100	99	100
100	37	92	100	99	98	98	100
1,000	15	25	79	95	26	42	79
10,000	15	16	27	45	17	22	30

* Evelyn colorimeter, 660 mμ filter, uninoculated medium = 100

sodium and ammonium ions, the "potassium requirement" observed represented not only that required for metabolic processes, but also that required to overcome the inhibitory action of Na^+ and NH_4^+ The requirement for potassium, as determined in the present medium, is about one-fifth of that previously observed It is not at all certain, however, that this represents solely the metabolic requirement for this ion Other ions in the present complex basal medium may enhance the requirement for K^+, just as did Na^+ and NH_4^+ in the previously used medium

It was of interest to determine whether the triethanolamine ion, which replaces Na^+ as the principal neutralizing cation in this medium, interfered to any extent with the utilization of K^+ For this purpose, extra triethanolamine was added to the medium as the hydrochloride, with results shown in Table V Since 175 to 200 mg of triethanolamine per 10 cc are used in the preparation of the medium, the amounts added do not represent the total concentration of this base in the medium Only after 200 mg of triethanolamine have been added in addition to that already present in the basal medium is there any appreciable toxicity for *Lactobacillus arabinosus* Since significant increases in triethanolamine concentration have so little effect on the response to K^+, it is reasonable to assume that the amounts originally present in the medium interfere very little in the

TABLE V

Effect of Triethanolamine on Response of Lactobacillus arabinosus to K^+

K^+ per 10 cc	Mg triethanolamine per 10 cc *				
	0	100	200	300	400
	Per cent of incident light transmitted†				
γ					
0	90	91	98	100	100
50	66	65	69	73	97
100	52	53	58	59	90
1000	21	22	23	27	44

* The amounts indicated are in addition to the triethanolamine used in the preparation of the medium (about 175 to 200 mg per 10 cc , see the text)

† As in Table IV

utilization of this ion At high concentrations of the amine, however, some degree of antagonism does exist between K^+ and the triethanolamine ion, since inhibition at low concentrations of K^+ is almost completely reversed at high At still higher concentrations of triethanolamine, however, the inhibition is not prevented by K^+

Rubidium-Potassium Relationship—The response of the five lactic acid bacteria to additions of Rb^+ to the potassium-free basal medium was determined On a molar basis, Rb^+ is slightly more active in promoting growth of *Streptococcus faecalis* than K^+ at low concentrations and slightly less so at high (Fig 4) The results of similar experiments with *Lactobacillus casei* and *Leuconostoc mesenteroides* 8042 are presented in Fig 5 For *L casei*, Rb^+ and K^+ are equally active at low concentrations, at higher concentrations, Rb^+ again is less active *L mesenteroides* 8042, by contrast, is unable to utilize Rb^+ in place of K^+. In similar experiments, *L arabinosus* and *L mesenteroides* 9135 proved intermediate in behavior For

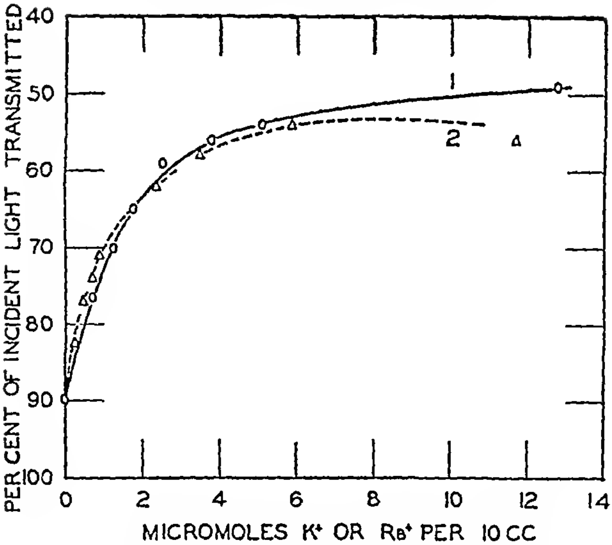

FIG 4 Comparative effects of Rb^+ and K^+ on growth of *Streptococcus faecalis* Curve 1, K^+, Curve 2, Rb^+

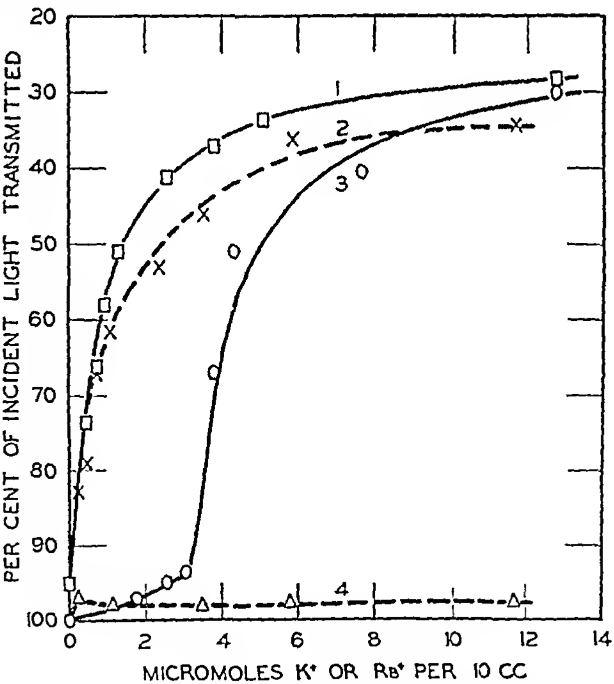

FIG 5 Comparative effects of Rb^+ and K^+ on growth of *Lactobacillus casei* and *Leuconostoc mesenteroides* 8042 Curves 1 and 2, response of *L casei* to K^+ and Rb^+ respectively, Curves 3 and 4, response of *L mesenteroides* to K^+ and Rb^+ respectively

these organisms, Rb^+ partially replaced K^+, but the response was erratic More than half maximum growth for either organism was never achieved with Rb^+ and high concentrations were inhibitory Thus, depending upon the organism, Rb^+ is able to replace K^+ completely, partially, or not at all

High concentrations of Rb^+ are inhibitory for *Leuconostoc mesenteroides* 8042 and the inhibition is reversed by the addition of sufficient K^+ (Table VI) A similar relationship was found with *L arabinosus*, despite the fact that at low concentrations Rb^+ partially replaces K^+ In organisms for which it is not the nutritional equivalent of K^+, Rb^+ thus acts in a manner analogous to Na^+ and NH_4^+

TABLE VI

Effect of K^+ on Inhibitory Action of Rb^+ for Leuconostoc mesenteroides 8042

K^+ per 10 cc	Mg Rb^+ per 10 cc			
	0	10	20	30
	Per cent of incident light transmitted*			
γ				
0	97	90	93	93
500	29	60	84	89
1,000	28	45	64	84
20,000	30	30	30	31

* As in Table IV

TABLE VII

Effect of Rb^+ on Inhibitory Action of NH_4^+ for Lactobacillus casei

Rb^+ per 10 cc	Mg NH_4^+ per 10 cc		
	0	20	40
	Per cent of incident light transmitted*		
γ			
0	67	89	90
100	46	78	89
500	29	51	66
1,000	25	47	56
10,000	33	24	25

* As in Table IV The antibacterial index ($[NH_4^+]/[Rb^+]$) for half maximum growth calculated from these data is about 350

For organisms which utilize Rb^+ in place of K^+, factors which interfere with the response to K^+ would be expected to interfere similarly with the response to Rb^+ The data of Table VII show that the inhibitory action of NH_4^+ for *Lactobacillus casei* is alleviated by Rb^+, just as it is by K^+

The ratio of NH_4^+ to K^+ permitting half maximum growth is about 230 (Table III), the corresponding ratio of NH_4^+ to Rb^+ is about 350 This indicates that NH_4^+ is more toxic in the presence of K^+ than in the presence of the same molar concentration of Rb^+, *i e*, Rb^+ is somewhat more effective in overcoming the inhibitory action of NH_4^+ than is K^+ This must indicate a greater affinity of this metallic ion for the enzyme with which it functions than K^+ possesses

TABLE VIII

Effect of K^+ on Inhibitory Action of Cs^+ for Lactobacillus arabinosus

K^+ per 10 cc	Mg Cs^+ per 10 cc			
	0	10	30	50
	Per cent of incident light transmitted*			
γ				
0	80	95		
500	25	24	38	82
1,000	19	23	26	43
10,000	20	20	21	22

* As in Table IV

TABLE IX

Effect of K^+, Na^+, and NH_4^+ on Li^+ Toxicity for Lactobacillus arabinosus

	Mg per 10 cc				
K^+	0	100	10	10	10
Na^+	0	0	10	0	10
NH_4^+	0	0	0	10	10
Mg Li^+ per 10 cc	Per cent of incident light transmitted*				
0	94	32	32	32	26
10	98	96	65	33	32
15	98	98	95	57	35
20	98	97	96	97	72

* As in Table IV

Cesium-Potassium Relationship—Cesium ion did not replace K^+ for the five organisms tested For *Lactobacillus arabinosus*, Cs^+ was inhibitory at high concentrations This inhibition was completely reversed by the addition of sufficient K^+ (Table VIII)

Lithium-Potassium Relationship—For *Lactobacillus arabinosus*, Li^+ was considerably more toxic than either Na^+ or NH_4^+ This toxicity was not alleviated by K^+ The addition of Na^+ relieved the inhibition slightly, NH_4^+ was somewhat more effective, and the two together were more effective than either alone (Table IX) These results suggest that both Na^+

and NH_4^+ ions play some essential metabolic rôle in *Lactobacillus arabinosus*, and that part of the toxic effects of Li^+ results from interference with these processes No direct evidence has been obtained to show that Na^+ is essential in the metabolism of these organisms It is obvious, however, that NH_4^+ must play a rôle in intermediary metabolism in these bacteria, and some direct evidence for this, obtained by growth effects (4, 5) exists

DISCUSSION

The term "ion antagonism" has been used to describe the opposite effect of ions or the effect of an "inactive" ion in removing the effect of an "active" one (6) The phenomenon was first observed by Ringer (7), who found that a solution of sodium chloride does not maintain the beat of a heart perfused with it unless additions of Ca^{++} and K^+ chlorides are made The concept of ion antagonism was applied to animals by Loeb (8), to plants by Osterhout (9), and to bacteria by Flexner (10), Eisler (11), and Lipmann (12) Since these early investigations, many further observations regarding the phenomenon have been reported (6)

Although it has been recognized recently that animals may be deficient in one mineral relative to an excess of some other (13), none of the early data on ion antagonism was able to relate the phenomenon to the inorganic requirements of the organism under investigation Loeb discounted the possibility that physiologically balanced solutions had nutritional significance for a number of organisms and favored the hypothesis that such solutions were required to maintain proper cell permeability (14)

The antagonistic effects of ions observed in this investigation are most easily explained as examples of the competitive interference of one ion with the essential rôle of another. This conclusion is based on the following observations (1) the inhibition is reversed only by an inorganic ion which is an essential metabolite for the microorganism, (2) the inhibition is apparent only in the presence of limiting concentrations of the metabolite and is reversed, in most cases completely, if the metabolite concentration is raised sufficiently, (3) the ratio of antagonist to metabolite which permits a given amount of growth is relatively constant over a considerable range of concentrations

It is usually assumed that inhibitions of this type result from the reversible combination of a metabolite or its analogue with some active surface or enzyme in or on the cell whose proper functioning is essential for growth Whether enzyme is present as the active enzyme-metabolite complex or as the inactive enzyme-antimetabolite complex thus depends upon the relative concentration of metabolite to antimetabolite, and not upon their absolute concentrations The factors affecting the affinity of a metallic ion for an enzyme are obscure The fact that Rb^+ can replace K^+ for

one organism and antagonize its action for another indicates subtle though critical differences in the structure of enzymes having the same or a similar function In both cases, however, the combination of Rb^+ with the enzyme is indicated That Rb^+ can substitute for K^+ in isolated enzymatic processes dependent upon the presence of the latter ion was shown by Lwoff and Ionesco (15), who showed that either Rb^+ or Cs^+ could function in place of K^+ in permitting production of pyruvate from malate by cell preparations of *Moraxella lwoffi*

Previous figures reported as the K^+ requirement for maximum growth of lactic acid bacteria ranged from 1 to 10 mg per 10 cc (1) On the basal medium used here, in the absence of Na^+ or NH_4^+, maximum growth can be obtained with amounts of K^+ ranging from 0 2 to 1 mg per 10 cc It is apparent that the concentration of an essential ion required for growth, or the concentration of an ion which will inhibit growth, is not a constant but may vary widely, depending upon the composition of the medium in which the determinations are made

The practical significance of these observations should not be overlooked Considerable and variable quantities of Na^+ and NH_4^+ are present in media used for microbiological assay of vitamins and amino acids Furthermore, if sodium chloride is added with a hydrolyzed sample, the amount present will vary from one assay tube to another In the absence of sufficient amounts of K^+, inhibitory concentrations of Na^+ may well be reached The use of potassium hydroxide in place of sodium hydroxide for neutralization of various components of the medium might be expected to eliminate these sources of error Such a medium was found to support excellent growth of the five organisms used in this investigation Observations concerning the relative toxicities of sulfate and chloride ions suggest that sulfuric acid might prove superior to hydrochloric acid in the preparation of samples for assay

SUMMARY

Preparation of a medium which is deficient in K^+ and relatively free of NH_4^+ and Na^+ is described Triethanolamine was used for neutralization of the acidic components of this medium The medium supports excellent growth of the five lactic acid bacteria tested when supplemented with adequate amounts of K^+

In this medium, it was shown that the magnitude of the K^+ requirement of all organisms investigated was greatly increased by the addition of Na^+ and NH_4^+ Whether or not these ions inhibited growth depended upon the ratio of their concentrations to that of K^+, and not upon the absolute amounts present For a given amount of growth these ratios were relatively constant over a fairly wide range of concentrations, *i e*, a competitive

relationship between these ions and the essential metabolite, K^+, appeared to be present

Rubidium ion replaced K^+ completely for growth of *Lactobacillus casei* and *Streptococcus faecalis*, partially for *L mesenteroides* 9135 and *L. arabinosus*, and not at all for *Leuconostoc mesenteroides* 8042 For the latter two organisms Rb^+ was inhibitory at higher concentrations and the inhibition was competitively alleviated by K^+. For *L casei*, which utilizes Rb^+ in place of K^+ for growth, Rb^+ was slightly more effective than K^+ in reversing inhibition of growth by NH_4^+

Cesium ion did not replace K^+ as an essential metabolite for any of these organisms For *Lactobacillus arabinosus*, the only organism investigated, Cs^+ was inhibitory at high concentrations, and this inhibition was alleviated by K^+

In contrast to the other alkali metal ions, toxicity of Li^+ for *Lactobacillus arabinosus* is not related to the K^+ requirement of the organism Inhibition of growth by Li^+ is prevented over a narrow range of concentrations by the addition of small amounts of NH_4^+ and Na^+

Certain of these results have been discussed briefly

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A SIMPLE APPARATUS FOR THE SERIAL MEASUREMENT OF THE RESPIRATORY EXCHANGE IN THE RAT

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(Received for publication, June 18, 1948)

For the frequent measurement of the respiratory exchange of small animals over successive short periods of time, all of the methods described present difficulties In the open circuit method determination of oxygen consumption over a short interval of time is not practical In the closed circuit methods considerable time is required for temperature equilibrium to be attained before a measurement can be made Both carbon dioxide and oxygen analyses are necessary in the closed non-ventilated chamber method[1]

In the method described here neither the maintenance of a constant temperature nor analysis for oxygen is required, thus facilitating the measurement of the respiratory exchange over successive short time intervals This method is based on a principle which, to our knowledge, has never been described before

The apparatus is a closed, non-ventilated chamber in which atmospheric pressure is maintained by means of a small spirometer For the calculation of the respiratory exchange, the data needed are the initial and final temperatures, barometric pressure, and the percentage of carbon dioxide In addition, the free volume of the chamber must be known and the system must be maintained at 100 per cent humidity The oxygen consumption is calculated as the carbon dioxide production plus the change in volume of the system

Fig 1 is a schematic diagram of the system *A* is a glass desiccator having three outlets in its cover, the free volume of this chamber is 3300 cc *B* is a small fan, *C* and *F* in Fig 1 are outlet and inlet tubes for flushing the chamber, *D* is the power supply cable for the fan motor, *G* is an iron-constantan thermocouple for measurement of the rectal temperature of the rat, and *H* is a thermometer (0–50°) readable to 0 02° S_1 is the 2-way cock in the capillary tube to the carbon dioxide gas analyzer whose accuracy is 0 02 per cent Tube *E* leads to the water manometer (*J*) and the small test-tube spirometer (*K*), which has a volume of about 70 cc and is counterbalanced by the weight (*L*) The spirometer is connected through a 2-way

[1] Benedict, F G , in Abderhalden, E , Handbuch der biologischen Arbeitsmethoden, Berlin and Vienna, Abt IV, Teil 10, 427 (1924)

cock (S_2) to a 50 cc glass syringe (M) which is read to 0 5 cc Preliminary to a series of determinations approximately 5 cc of 10 per cent sulfuric acid are placed in the desiccator to obviate the absorption of any appreciable amounts of carbon dioxide

The respiratory exchange is determined by the following procedure. The rat is placed on a perforated porcelain support in the desiccator and the cover is affixed [2] After E is clamped off and S_1 is closed, air saturated with water at 36° is blown through the chamber at about 25 liters per minute through F and exhausted at C This rate of flushing is sufficient to prevent any detectable accumulation of carbon dioxide During the process of flushing the counterpoise is disconnected and the spirometer is brought to

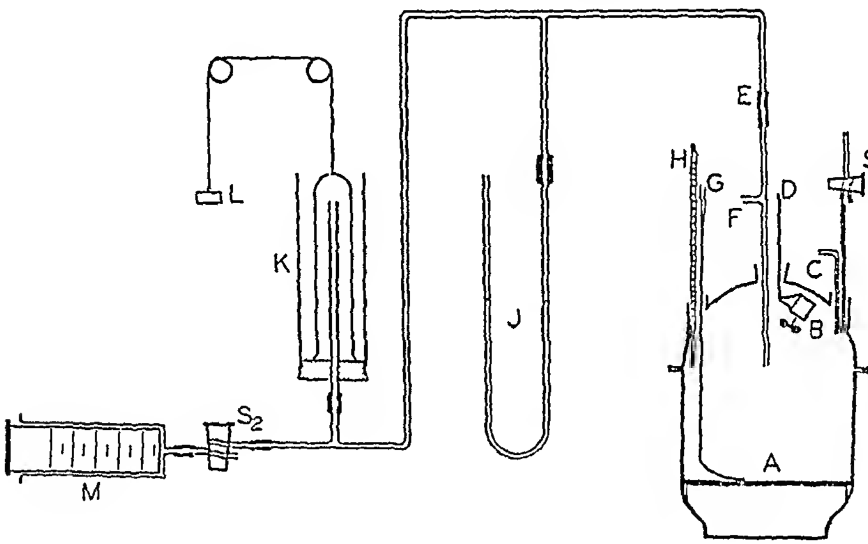

FIG 1 Schematic diagram of the respiratory apparatus

its lowest level and adjusted to atmospheric pressure with the syringe When the manometer indicates atmospheric pressure, S_2 is turned to room air and 50 cc of air are admitted into the syringe The room temperature and the volume of air are recorded S_2 is then connected with the spirometer and the air in the syringe is admitted to it At the time chosen to start the experimental period, the air flow is stopped, F and C are clamped, E is unclamped, and the temperature of the chamber and the barometric pressure are recorded

About $1\frac{1}{2}$ minutes before the period is over the fan is started to attain temperature equilibrium Some 7 seconds before the end of the period the fan is stopped and the spirometer is brought to atmospheric pressure by

[2] Dow-Corning high vacuum type Silicone stop-cock grease gives an air-tight seal more conveniently than other greases tried

means of the syringe or the counterpoise At the end of the period, E is clamped and the temperature of the chamber and the barometric pressure are read The fan is started again, S_1 is opened, and a sample of air from the chamber is drawn for carbon dioxide analysis The time elapsed between the end of the experimental period and the final drawing of the air sample is noted The fan is stopped, S_1 is closed, C and F are unclamped, and the flushing is begun again The volume of air left in the spirometer is measured by the syringe and this volume and the room temperature recorded

50 cc of air are again admitted into the spirometer The analysis for carbon dioxide is performed and recorded and the apparatus is now ready for another determination During the second experimental period the results of the first period may be calculated

With a room temperature of approximately 24° the temperature of the chamber is maintained close to the critical temperature of the rat (28°) During an experimental period the temperature changes no more than 0 4° The data obtained are recorded as follows b p = barometric pressure, V_{ch} = free volume of chamber, T^1_{ch}, T^2_{ch} = initial and final temperature in chamber, T^1_{rm}, T^2_{rm} = initial and final temperature of room, V^1_s, V^2_s = initial and final volume of syringe, and W^1_{ch}, W^2_{ch}, W^1_s, W^2_s = vapor pressure of water in chamber and spirometer at observed temperatures The dry gas volumes at standard temperature and pressure initially (V^1) and finally (V^2) are

$$V^1 = \left[\frac{\text{b p} - W^1_{ch}}{T^1_{ch} + 273\ 18} \times V_{ch} \times \frac{273\ 18}{760}\right] + \left[\frac{\text{b p} - W^1_s}{T^1_{rm} + 273\ 18} \times V^1_s \times \frac{273\ 18}{760}\right]$$

and V^2 is similarly calculated The percentage of carbon dioxide found (less 0 03 per cent for that present in atmospheric air) is adjusted for the time period used If the period is 15 minutes and the air sample from the chamber is obtained at 15 minutes and 30 seconds, the percentage of carbon dioxide produced per 15 minutes is 15/15 5 of that actually found by analysis The amount of carbon dioxide produced is then

$$\text{Cc } CO_2 = \%CO_2 \times V^2$$

and the amount of oxygen consumed is

$$\text{Cc } O_2 = \text{cc } CO_2 + (V^1 - V^2)$$

If the R Q is unity, there will be no change in volume of the system, since equal amounts of carbon dioxide are produced and oxygen consumed and V^1 will be equal to V^2 If the R Q is less than unity, V^2 will be less than V^1 by an amount equal to the difference between the oxygen consumed and the carbon dioxide produced

The time-separating experimental periods need be no longer than 5 minutes, so that three 15 minute periods of oxygen consumption and carbon dioxide production may be measured by one operator in 1 hour and the requisite calculations performed

As in all measurements of the respiratory exchange of small animals, the system must be air-tight This feature is tested occasionally by increasing and decreasing the pressure within the system by means of the syringe (the spirometer being clamped off) and observing the manometer In addition a blank run is conducted, the calculated V^1 should not differ from V^2 by more than about 0 2 per cent

The accuracy of the entire procedure was tested by the combustion of two pure, organic compounds For this purpose the thermocouple was replaced by a power cable connected within the chamber to a short length of platinum wire The platinum wire is placed in contact with a glass wool

TABLE I

Ethyl Alcohol

R Q = 0 667

CO_2 produced	O_2 consumed	R Q	Error in R Q
cc	*cc*		*per cent*
79 5	119 8	0 664	−0 40
85 6	131 8	0 649	−2 65
83 7	126 2	0 663	−0 55
175 6	268 5	0 654	−1 90
165 0	251 3	0 657	−1 45
192 5	291 5	0 660	−1 00

wick which protrudes about 2 mm from a short length of heavy capillary tubing (7 mm outer diameter × 20 mm) The capillary is held in glass to glass contact by means of a short length of rubber tubing with a small shell vial (9 mm outer diameter × 30 mm) which holds the liquid to be combusted and into which the wick extends The combustion lamp is held in place within the chamber by means of a rubber stopper The calculated volume of these appurtenances is subtracted from the free volume of the chamber

The procedure for measurement is essentially the same as that described for the rat The lamp is lighted by momentarily heating the platinum to just less than white heat after the chamber has been sealed at S_1, C, and F and opened at E The spirometer is left at its lowest level to allow for the initial, rapid expansion of volume that takes place from the rise in temperature as a result of the combustion The combustion can be terminated at any time by starting the fan. The amount of material that can be

burned is determined by the initial concentration of oxygen To burn greater amounts of material, air enriched in oxygen (about 28 per cent oxygen) and saturated with water is flushed through the chamber After the lamp is extinguished, the temperature within the chamber is allowed to fall to within 0 5° of the initial temperature before the final readings are made This insures complete saturation within the chamber

With this technique, 95 per cent ethyl alcohol was burned, the results are shown in Table I The loss of ethyl alcohol, by volatilization, is great, due to the flushing of the chamber and heating of the platinum This precludes calculation of the theoretical oxygen consumption, but not of the R Q No error is introduced by the evaporation of ethyl alcohol, since

TABLE II

Diethylene Glycol

R Q = 0 800

Amount combusted	CO_2 produced	O_2 consumed	R Q	Error in CO_2	Error in O_2	Error in R Q
mg	*cc*	*cc*		*per cent*	*per cent*	*per cent*
116 6	97 0	124 2	0 781	−1 52	+0 89	−2 37
118 8	98 8	128 4	0 769	−1 59	+2 31	−3 87
303 0	250 2	320 0	0 782	−2 27	0 00	−2 25
257 7	212 4	271 1	0 783	−2 43	−0 37	−2 11
137 0	119 1	153 1	0 778	+2 94	+5 81	−2 75
284 9	243 3	310 1	0 785	+1 08	+3 06	−1 87
303 2	255 7	323 9	0 789	−0 20	+1 16	−1 37
66 9	55 4	69 2	0 801	−1 95	−2 12	+0 13

practically all of it is dissolved in the water of the chamber and does not appear as gas

Diethylene glycol was used in a similar test of the apparatus since none is lost by volatilization, the results obtained are given in Table II

The errors observed are insignificant in the calculation of physiological data

SUMMARY

A non-ventilated closed chamber is described for the measurement of the respiratory exchange of the rat By maintaining the chamber at atmospheric pressure the oxygen consumption may be calculated from an analysis for carbon dioxide and the change in the volume of the system No time-consuming temperature equilibration of the system is required With this simple apparatus one operator may perform serial measurements of short duration of the respiratory exchange

MICROBIOLOGICAL DETERMINATION OF ARGININE IN PROTEINS AND FOODS

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(Received for publication, May 12, 1948)

Lactobacillus casei (1-4), *Lactobacillus arabinosus* (5), *Leuconostoc mesenteroides* P-60 (6-7), *Lactobacillus fermenti* 36 (8), and *Streptococcus faecalis* (9-14) have all been proposed or used for the assay of arginine The assays reported in this paper were made with *Streptococcus faecalis* and with the medium previously used for threonine, valine, and histidine (15-17)

EXPERIMENTAL

Streptococcus faecalis 9790[1] was employed in the assays described

Basal Medium—The basal medium was the same as that described in a previous paper for methionine (18), with the exception that pyridoxine was replaced by 400 γ of pyridoxamine

Assay Procedure—The procedures followed for the cultures, inoculum, and preparation of samples were identical with those described in other papers (15, 18)

Preparation of Arginine Standards—Suitable concentrations of L-arginine monohydrochloride were used for the development of the standard curve The titration values on the standard curve (Fig 1) were not altered by the addition to the medium of 1 2 mg of any of the other nineteen amino acids

The recovery of arginine added to hydrolysates of casein, whole corn, rye, and zein gave results well within the experimental error for this type of assay (Table I)

Table II shows values found for several foods and proteins at different assay levels Data on the reproducibility of values found for nine materials of diverse character are shown in Table III

The results (Table IV) found for the proteins and foods[2] agree well with other microbiological methods

The values reported by Vickery (19) on six proteins and those of Bergdoll and Doty (21) on four proteins are in close agreement with our results

[1] Obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D C

[2] The sources and preparation of the samples assayed are given in a previous publication on the determination of methionine (31)

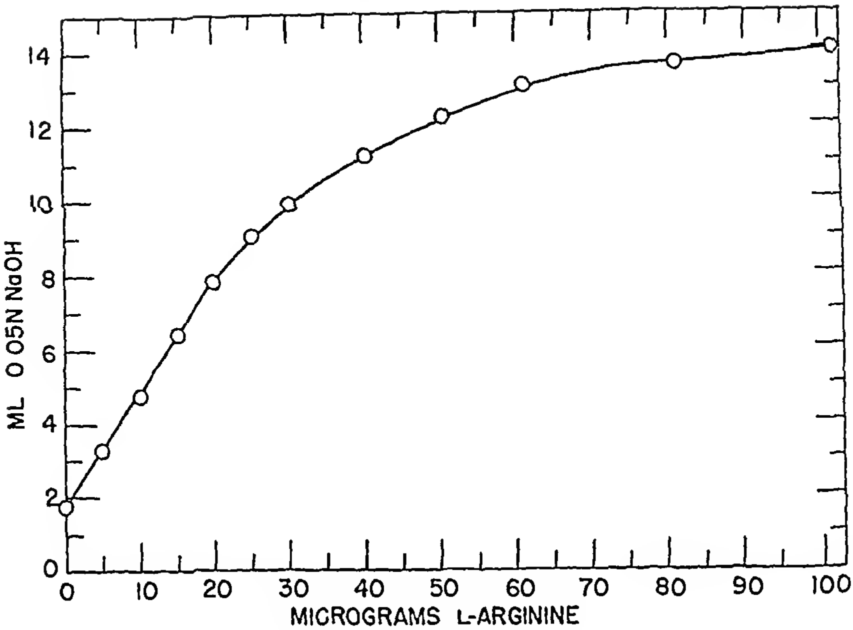

FIG 1 Standard titration curve

TABLE I

Recovery of Arginine Added to Protein Hydrolysates

Protein hydrolysate	Arginine				
	In hydrolysate*	Added	Total	Found	Recovery
	γ	γ	γ	γ	*per cent*
Casein	3 50	20	23 50	23 50	100
	7 00	20	27 00	27 60	102
	10 50	20	30 50	30 70	101
	14 00	20	34 00	34 00	100
Corn, whole, yellow	5 50	5	10 50	10 00	95
	5 50	10	15 50	15 30	98
	5 50	15	20 50	20 80	101
	5 50	20	25 50	25 70	101
Rye, whole	5 90	10	15 90	15 80	99
	11 80	10	21 80	22 00	101
	17 70	10	27 70	27 70	100
Zein	3 50	5	8 50	8 00	94
	3 50	10	13 50	13 50	100
	3 50	15	18 50	18 50	100
	3 50	20	23 50	24 00	102

* Not corrected for moisture and ash

Vickery used flavianic acid and Bergdoll and Doty a chromatographic separation in assaying this amino acid

TABLE II

*Arginine Content at Different Assay Levels**

Material	Level added	Found	Material	Level added	Found
	γ	per cent		γ	per cent
Arachin	100	12 5	Soy bean flour	200	3 85
	200	12 5		400	4 00
	300	12 1		600	3 70
	400	12 5		800	3 85
Average		12 4			3 85
Milk, dry, skim	200	0 95	Wheat bran globulin	100	12 70
	400	0 95		200	12.75
	600	0 95		300	12 67
	800	0 96		400	12 75
Average		0 95			12 72
Oatmeal	500	1 10	Wheat germ, defatted	200	2 50
	1000	1 15		400	2 43
	1500	1 13		600	2 50
	2000	1 14		800	2 48
Average		1 13			2 48
Rye, whole	1000	0 60			
	2000	0 60			
	3000	0 60			
	4000	0 60			
Average		0 60			

* Not corrected for moisture and ash

TABLE III

*Reproducibility (Per Cent) of Arginine Content When Determined by Separate Assays**

Material	Assay 1	Assay 2	Average
Casein	3 50	3 56	3 53
Conarachin	15 61	15 37	15 49
Corn, whole, yellow	0 54	0 55	0 55
Cottonseed flour	6 67	6 85	6 76
Egg, whole, dried	4 28	4 39	4 33
Milk, dry, skim	1 01	0 96	0 99
Peanut, total globulins	13 25	13 36	13 30
Rye, whole	0 60	0 58	0 59
Zein	1 82	1 90	1 86

* Not corrected for moisture and ash

SUMMARY

A microbiological method is described for the determination of arginine in proteins and foods with *Streptococcus faecalis*. The results of assays on

TABLE IV

Arginine Content of Some Proteins and Foods

Percentages calculated for ash- and moisture-free material

Material	N	Arginine	Values from literature
	per cent	*per cent of food*	
Arachin	18.30	13.50	13.94 (19), 15.4 (20)
Casein	16.07	3.81	3.7 (2), 3.6 (4), 3.7 (7), 3.9 (10), 3.6 (11), 3.72 (19), 3.7 (21), 4.3 (20), 3.60 (22)
Coconut globulin	17.42	16.73	15.92 (23)
Conarachin	18.20	16.53	12.7 (20)
Cottonseed globulin	18.00	14.72	14.92 (19), 13.6 (24)
Edestin	18.55	16.51	16.7 (13), 16.76 (19), 14.0 (20)
Gelatin (Bacto)	18.32	9.11	9.30 (2), 8.0 (4), 9.1 (10), 8.68 (19), 9.08 (21)
Glycinin	17.30	7.94	8.5 (20)
Lactalbumin	15.39	3.42	3.2 (4), 3.4 (21)
Ovalbumin (crystalline)	15.98	6.03	6.6 (2), 5.7 (7), 5.94 (10), 5.66 (19), 6.0 (21)
Ox muscle	16.00	7.87	6.0 (4), 6.3 (20), 6.9 (25)
Peanut, total globulins	18.01	14.16	
Phaseolin (navy bean)	16.07	5.97	
Wheat bran globulin	17.76	13.30	
Zein	16.00	1.95	1.60 (19), 1.80 (20)
Barley, pearled	1.86	0.53	0.32 (14)
Brazil nut meal	9.03	7.62	
Corn germ, defatted	3.93	2.18	1.67 (20), 2.48 (26)
" whole, yellow	2.22	0.65	0.59 (14), 0.56 (20), 0.46 (27)
Cottonseed flour	10.36	7.72	4.87 (14), 4.79 (20), 8.55 (26)
Egg, whole, dried	8.11	4.82	3.24 (28)
Milk, dry, skim	6.57	1.15	1.48 (10), 1.31 (11), 1.24 (14)
Oatmeal	2.73	1.26	0.70 (14), 1.02 (20)
Peanut flour	10.15	7.76	6.29 (20), 8.75 (26)
Peas, black-eyed	4.15	1.95	
Rice, white	1.26	0.69	0.57 (20)
Rye, whole	1.98	0.67	0.53 (10)
Soy bean flour	8.85	4.33	3.93 (10), 2.92 (14), 4.15 (29)
Wheat germ, defatted	6.50	2.80	2.44 (20), 3.90 (26)
" whole	3.07	0.86	0.81 (10), 0.59 (14), 0.63 (20)
Yeast, dried, brewers'	7.71	2.26	2.17 (10), 2.08 (30)

thirty-one proteins and foods agree closely with those obtained on the same materials by both chemical and microbiological methods

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THE AVAILABILITY OF DL-LANTHIONINE FOR THE PROMOTION OF GROWTH IN YOUNG RATS WHEN ADDED TO A CYSTINE- AND METHIONINE-DEFICIENT DIET

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(Received for publication, May 12, 1948)

In an earlier communication (1) it was reported that the internally compensated thio ether amino acid, mesolanthionine, is not utilized for the growth of young rats subsisting on a cystine- and methionine-deficient diet Two alternative explanations were given for this failure of the animals to utilize mesolanthionine, either that the animals were unable to split the thio ether or that cleavage did occur in such a way that the unutilizable D-cysteine (2) was formed instead of L-cysteine In order to develop information on this question, feeding experiments have now been conducted with DL-lanthionine By feeding the racemic form under the same conditions by which the mesolanthionine was fed, it was believed that evidence could be obtained to show which of the above alternative explanations for the failure of mesolanthionine to support growth is correct If the L component of the DL-lanthionine were split, one-fourth of the DL-lanthionine would supply L-cysteine irrespective of what side of the sulfur in the molecule cleavage occurred The results of the experiments here reported show that DL-lanthionine supports growth to the extent that the L component is cleaved to yield 1 molecule of L-cysteine

DL-Lanthionine usually accompanies mesolanthionine in acid hydrolysates of alkali-treated proteins (3) The DL-lanthionine used in these experiments was obtained from acid hydrolysates of human hair that had been previously boiled for 1 hour with 2 per cent Na_2CO_3 solution [1]

EXPERIMENTAL

In the previous investigation (1) on the nutritional availability of mesolanthionine, to supply the B vitamins a cystine and methionine basal diet having the following percentage composition was satisfactorily used casein 6 0, dextrin 37 0, sucrose 15, salt mixture 4 (4), agar 2 0, lard 19, cod liver oil 5 0, and a commercial milk-vitamin concentrate 12 When fed this diet the animals invariably lost weight

[1] Tests made on the DL lanthionine for disulfide gave negative results, showing that it was free from any trace of cystine

Since the milk-vitamin concentrate previously used was no longer available, a simpler basal diet containing synthetic vitamins and choline was first employed. It was found, however, that with 6 per cent casein in the diet, as previously used with the milk-vitamin concentrate, the animals grew too fast to make it possible to detect the effect of adding the amino acid supplements. By omitting choline and reducing the level of casein to 4.21 per cent, a satisfactory basal diet was obtained. When fed this diet, the animals declined rapidly in weight. Addition of the sulfur amino acids promptly arrested the decline in weight, and growth proceeded at a satisfactory rate. Salmon (5) has pointed out that the primary deficiency of casein at low levels is in labile methyl groups. This is in accord with the decline in weight of our rats when choline was omitted from the diet. However, this diet with a low content of fat, devoid of choline and containing 4.21 per cent of casein, supported growth at a satisfactory rate after it had been supplemented with cystine as well as with choline, methionine, or DL-lanthionine. Unless the cod liver oil or corn oil used in the diet contained an appreciable amount of choline, the methionine in the casein may have supplied enough methyl groups to be adequate for growth when cystine was added.

The data presented represent the results obtained with eleven lots of albino rats. In addition to these, several lots were used in preliminary work on developing a satisfactory basal diet. Each lot consisted of four or six animals from the same litter, equally divided with respect to sex and having initial weights of 50 to 60 gm. They were housed in individual cages having wide mesh screen bottoms and kept in a room maintained at about 24.4°. The animals were weighed twice weekly. After considerable experimentation a basal diet having the following percentage composition was found satisfactory: casein (Labco) 4.21, dextrinized corn-starch 90.79, salt mixture 2.00 (4), cod liver oil 2.00, and corn oil 1.00. A vitamin mixture was incorporated in the dextrinized corn-starch which provided the following constituents in each 100 gm. of diet: thiamine hydrochloride 0.2 mg., pyridoxine hydrochloride 0.2 mg., riboflavin 0.3 mg., calcium pantothenate 0.3 mg., and niacin 1 mg. These vitamins, in an aqueous-alcoholic solution, were added to the dextrinized starch and the mixture was dried at 50° to the original weight of the starch. The diet was fed *ad libitum* and a record of the food intake is given in Table I. It is of interest to note that in general the food consumption was definitely lower during the periods when the animals subsisted on the basal diet alone than when the amino acid supplements were incorporated.

The animals fed the basal diet alone invariably lost weight rapidly, and when the feeding was prolonged they died in about 45 days. For comparison with the behavior of the rats given DL-lanthionine, L-cystine and

DL-methionine were also used The addition of 0 3 gm of cystine or 0 37 gm of methionine to 100 gm of the basal diet made it effective in promptly arresting the decline in weight of the animals, and in enabling them to grow

TABLE I*

Food Consumption

Lot No	Rat No	Experimental period	Supplement to basal diet	Average daily food consumption
		days	*per cent*	*gm*
210	4 ♀	41	0 30 cystine	11 9
213	1 ♂	23	No supplement	6 6
		47	0 37 methionine	6 8
208	2 ♀	22	No supplement	4 9
		26	0 30 cystine	6 4
218	2 ♀	20	No supplement	5 7
		14	1 04 DL lanthionine	5 6
		17	No supplement	2 1
220	2 ♂	11	1 04 DL lanthionine	9 4
		8	No supplement	3 1
		6	1 04 DL-lanthionine	4 7
220	3 ♀	11	1 04 "	10 3
		8	No supplement	3 4
		6	1 04 DL lanthionine	5 5
211	1 ♂	40	0 37 methionine	9 3
212	4 ♀	24	No supplement	6 2
		16	0 30 cystine	7 0
		16	No supplement	6 3
		9	0 30 cystine	10 6
220	1 ♀	12	1 04 DL lanthionine	8 4
		7	No supplement	4 4
		7	1 04 DL lanthionine	4 7
218	4 ♀	18	No supplement	5 9
		14	1 04 DL lanthionine	6 6
		18	No supplement	4 0
218	3 ♂	20	" "	5 1
		13	1 04 DL lanthionine	4 4
		16	No supplement	3 9
218	1 ♂	42	" "	3 2

* This table includes the record of only those rats, the growth curves of which are shown in Figs 1 and 2

at a satisfactory rate Likewise, equally prompt and effective responses followed the addition of 1 04 gm of DL lanthionine to the basal diet The above-stated amounts of the three supplements added to the diet represent biologically available sulfur equivalents

In six of the lots all of the animals were fed the basal diet from the start

for periods of 15 to 25 days Following the fore periods on the basal diet the animals were fed one of the supplements In most cases, one rat of a lot was allowed to continue on the basal diet throughout the remainder of the feeding period to serve as a control In several lots the effect of the supplements was studied throughout successive periods on the same animal

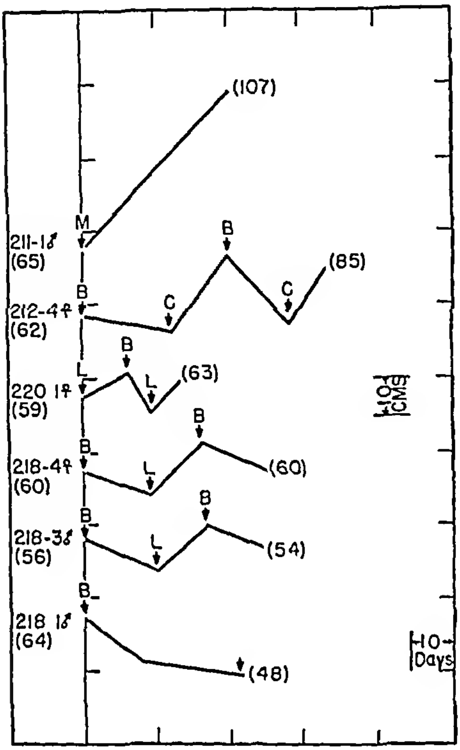

Fig 1

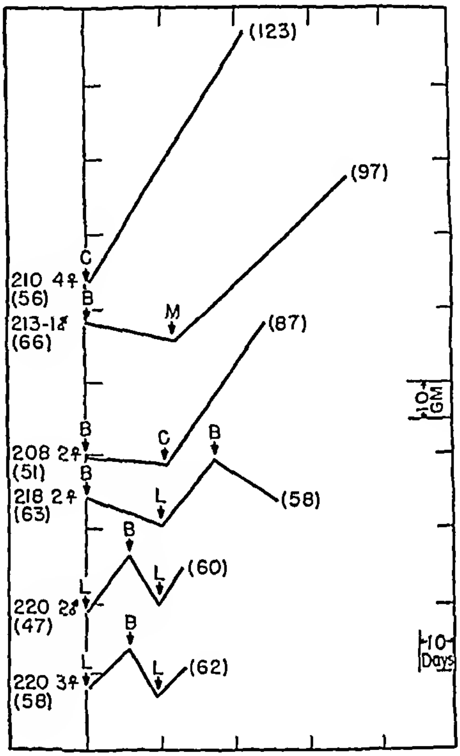

Fig 2

Figs 1 and 2 Growth curves of rats receiving the basal-deficient diet (Diet B) alone, or supplemented with 0 3 per cent of L-cystine (Diet C), 0 37 per cent of DL methionine (Diet M), or with 1 04 per cent of DL-lanthionine (Diet L) The introduction of each diet is indicated by the symbols over the downward arrows The identification number and sex of the rats are given on the extreme left with their initial and final weights in parentheses

Without exception, DL-lanthionine proved to be as effective as cystine or methionine in its capacity to promote growth at the start, or to restore it after a period of decline on the basal diet alone The growth curves for representative animals from the various lots are given in Figs 1 and 2 These are typical of the others which are omitted to save space

SUMMARY

Feeding experiments have shown that racemic lanthionine can replace cystine and methionine in the diet Young albino rats fed a low protein

(casein) basal diet deficient in cystine, but adequately supplied with the non-protein dietary essential factors, declined in weight rapidly For comparison with the behavior of rats given racemic lanthionine, L-cystine and DL-methionine were also fed The addition of 0 3 gm of L-cystine or 0 37 gm of DL-methionine per 100 gm of basal diet caused immediate resumption of growth The addition of DL-lanthionine likewise resulted in corresponding growth resumption An immediate decline occurred when the lanthionine was omitted from the basal diet, and growth again was resumed when the lanthionine was supplied Previous experiments similarly conducted with mesolanthionine showed that this isomer is not utilized for growth

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A NEW SYNTHESIS OF CYSTINE*

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(Received for publication, June 21, 1948)

The present paper describes a new and convenient synthesis of cystine from pyruvic acid The essential steps are illustrated in the following reaction scheme

$$CH_3COCO_2H + CH_3CONH_2 \rightarrow CH_2 = C(NHCOCH_3)CO_2H \quad (A)$$

$$(A) + CH_3COSH \rightarrow CH_2(SCOCH_3)CH(NHCOCH_3)CO_2H \quad (B)$$

$$(B) + H_2O + (O) \rightarrow (—SCH_2CHNH_2CO_2H)_2$$

Acetamidoacrylic acid is prepared from pyruvic acid and acetamide by the method of Bergmann and Grafe (2) As pointed out by previous investigators (2–4), α-acetamidoacrylic acid, an eneamide, can also have a tautomeric imide form, but the amide structure is believed to express better the behavior of the compound in the present cystine synthesis

Addition of thiolacetic acid to α-acetamidoacrylic acid is accomplished in the presence of a trace of ascaridole as catalyst, giving N,S-diacetyl-DL-cysteine, m p 118°, in a yield of 85 per cent of the theoretical N,S-Diacetylcysteine of unspecified optical character has been prepared previously by the action of ketene on cysteine and reported to melt at 111–112° (5) and at 109–110° (6) Hydrolysis of the diacetyl derivative with concurrent oxidation by iodine gives 72 per cent of the theoretical amount of cystine

This paper is based on work carried out under the supervision of Dr W A Lazier[1] for the Office of Scientific Research and Development under Contract OEMsr-377 with E I du Pont de Nemours and Company

EXPERIMENTAL

α-Acetamidoacrylic Acid—α-Acetamidoacrylic acid and α,α-bis(acetamido)propionic acid were obtained by heating pyruvic acid with acetamide, as described by Bergmann and Grafe (2) Warming the bisacetamido compound with acetic acid produced additional amounts of the desired acrylic derivative, as reported by these authors

N,S-Diacetyl-DL-cysteine—To a mixture of 1 78 gm of α-acetamido-

* Contribution No 237, see Farlow (1)

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acrylic acid and 10 ml of thiolacetic acid (7) was added a fraction of a drop of ascaridole, and the mixture was refluxed for 20 minutes until solution was complete, and then for an additional 15 minutes Evaporation of the reaction mixture in a vacuum desiccator gave a crystalline residue which was recrystallized from a mixture of chloroform and petroleum ether The recrystallized addition product melted at 118° and had a neutral equivalent of 203 as compared with the theoretical value of 205 for diacetylcysteine The yield was 2 41 gm (85 per cent)

Cystine—A solution of 1 03 gm of diacetylcysteine in 10 ml of concentrated hydrochloric acid was boiled gently and a 0 53 N solution of iodine in methanol was added from a burette in portions of about 0 2 ml , as rapidly as decolorized until a permanent color of iodine remained for 5 minutes after the last addition 10 ml of iodine solution, or about 105 per cent of the theoretical quantity, were required The addition covered a period of 30 minutes The solution was evaporated almost to dryness, diluted with water, and sodium acetate was added until the solution was alkaline to Congo red paper The cystine, which separated very slowly, was removed by filtration and washed with water, alcohol, and ether The dry product weighed 0 43 gm or 72 per cent of the theoretical

Analysis—$C_6H_{12}N_2O_4S_2$	Calculated	C 30 4, H 5 4, N 11 0, S 26 4
	Found	" 30 0, " 5 0, " 11 7, " 26 6

As further confirmation of its identity, the cystine was converted by sodium-ammonia reduction and alkylation into S-benzyl-DL-cysteine, and the latter by acetylation into S-benzyl-N-acetyl-DL-cysteine by the methods of du Vigneaud and coworkers (8) These derivatives were found to melt at 215–216° and 157°, respectively, as compared with the values of 215–216° and 158° reported by du Vigneaud

SUMMARY

A new synthesis of cystine is presented Pyruvic acid is treated with acetamide to give α-acetamidoacrylic acid, to which is added thiolacetic acid to produce N,S-diacetyl-DL-cysteine Hydrolysis and oxidation of the latter produce cystine

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A COLORIMETRIC METHOD FOR ESTIMATION OF DEHYDROISOANDROSTERONE AND ITS APPLICATION TO URINE EXTRACTS*

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(Received for publication, May 19, 1948)

Kerr and Hoehn (2), while investigating the specificity of a modified Pettenkofer reaction, observed that dehydroisoandrosterone (DHA) gave a strongly positive reaction The similar behavior of cholic acid had long been known, and a number of procedures for its quantitative estimation based on this color reaction have been described (3–5) We have found that the Schmidt and Hughes procedure (4) for the determination of cholic acid can be adapted to the photoelectric colorimeter and used for the quantitative determination of DHA Further studies on the specificity of the reaction for DHA, on a quantitative basis, have enabled us to make certain generalizations concerning the structural features necessary for a positive color test We have also made a preliminary investigation of the application of the color reaction to extracts of human urine and, with certain reservations which will be discussed, we believe that the color reaction provides a simple analytical method for DHA applicable to extracts from natural sources The method should be of value in the further investigation of the metabolism of DHA in normal and pathological individuals, particularly the rôle it may play as an intermediate in the metabolism of the adrenal cortical hormones

EXPERIMENTAL

Reagents—

1 *Furfural solution* A commercial grade of furfural is distilled twice on a boiling water bath under reduced pressure The middle fraction only is retained from each distillation For a satisfactory product it is essential

* Preliminary reports were presented at the American Association for the Advancement of Science Gibson Island conference on hormones, 1943 (1), and at the Technological Conference, Chicago Section of the American Chemical Society, January 24, 1947

This work was supported in part by a grant from Armour and Company and in part by the Dr Wallace C and Clara A Abbott Memorial Fund of the University of Chicago

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that the temperature of a boiling water bath is not exceeded The nearly colorless product is promptly dissolved in 50 per cent acetic acid at a concentration of 0 56 per cent (volume per volume) and stored in the cold We have found the reagent, stored at $-5°$, to be stable for many months

2 *DHA standard solutions* A solution containing 10 mg of DHA or 11 45 mg of DHA acetate per 100 ml of glacial acetic acid is prepared, and additional standards containing 0 060 and 0 020 mg of DHA per ml are obtained by dilution with glacial acetic acid In those experiments in which DHA was used as a standard, the substance melted at 139–141° (corrected), $[\alpha]_D^{21} = +10\ 4°$ (ethanol) Because DHA crystallizes in two polymorphic modifications, the melting point is an unsatisfactory criterion of purity We have therefore, for the greater portion of the work, used the acetate of this compound (m p 169–170 5° (corrected), $[\alpha]_D = +4\ 2°$ (ethanol)) as the standard but have expressed the results in terms of DHA The acetate gives the same intensity of color mole for mole as the free hydroxy ketone

3 *Sulfuric acid, 16 0 N*

4 *Acetic acid, 50 per cent by weight*

Provided all test solutions are treated uniformly, minor changes in reagent concentrations, temperature, and time are relatively unimportant The recommended concentrations of reagents differ from those of Schmidt and Hughes (4) in preparation, their use leads to the same concentrations in the final mixture as specified by these authors

An amount of pure steroid or of urine extract estimated to contain from 10 to 50 γ of DHA or its equivalent is transferred to a calibrated Evelyn colorimeter tube and evaporated to dryness on a water bath under a stream of nitrogen The dry residue, if the evaporation has been properly carried out, is confined to a small area in the bottom of the tube and is dissolved in 0 5 ml of glacial acetic acid, with warming if necessary The solutions in Table I are then added to Evelyn tubes in duplicate or triplicate and mixed

To each tube indicated in Table I, 7 5 ml of 16 N sulfuric acid are added at 1 minute intervals After mixing well, the tube is placed in an efficient, large capacity, constant temperature water bath maintained at 67 ± 0 2° After exactly 12 minutes in the bath, the tube is removed and immediately placed in an ice bath for 1 minute After all the tubes have been heated and cooled (a series of thirty to forty tubes can be analyzed conveniently), the color intensity is determined in the Evelyn colorimeter, with Filter 660, the wave-length of maximum absorption of the colored product A center setting is obtained by adjusting the "blank without furfural" to 100 Since all the solutions containing furfural increase slightly in color while standing at room temperature, the colorimetric measurements are made

at approximately the same time interval (±10 minutes) after removal from the bath

There is a small but significant day to day variation in the color intensity developed by DHA standards, even though the same reagents are used and the assay conditions are apparently identical. It is therefore essential that a full set of standards be included in each assay series. The color produced is affected significantly by changes in furfural concentration, H_2SO_4 concentration, bath temperature, and heating time. Therefore it is also essential to use the same reagents and assay conditions for standards, blanks, and extracts

TABLE I
Reagents for Dehydroisoandrosterone Determination

	Glacial acetic acid	DHA standard solution	50 per cent acetic acid	Furfural solution	Urine extract in glacial acetic acid
	ml	*ml*	*ml*	*ml*	*ml*
Blank without furfural	0 5		2 0		
Reagent blank	0 5			2 0	
DHA standards (10, 30, and 50 γ levels)		0 5		2 0	
Urine extract blank			2 0		0 5
" "				2 0	0 5

Calculations

1 The galvanometer readings (G) are converted to L values ($L = 2 - \log G$. A convenient table for conversion is included in the manual accompanying the Evelyn colorimeter) If replicate determinations have been made, the mean L value for each set of replicates is calculated

2 To eliminate non-specific color (a) originally present in the extract and (b) produced by the action of H_2SO_4 alone on the extract, the L value for the "urine extract blank" is subtracted from that of the "urine extract" Occasional urine extracts develop a slight turbidity, which also is corrected by the urine extract blank. The mean L values of the reagent blank and the DHA standards are plotted on graph paper and a curve drawn connecting the points (The curve deviates slightly from strict linearity, thus differing from that obtained in the analysis of cholic acid) The DHA content of the urine extract aliquot is estimated by interpolation on the graph, and the total DHA content of the extract is obtained by application of the appropriate factor. An example of a standard curve is shown in Fig 1, and a sample set of calculations is given in Table II

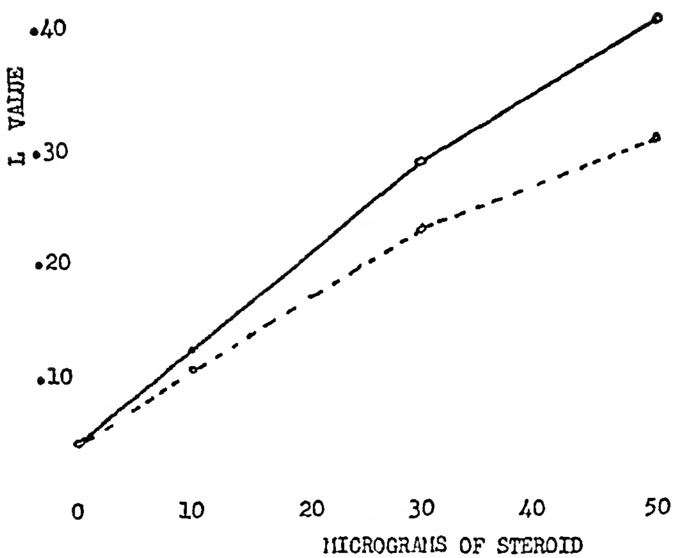

FIG 1 Calculation of standard curve $L = 2 - \log G$ The solid curve represents dehydroisoandrosterone or 3(β)-chloroandrostenone-17, the dotted curve $\Delta^{3,5}$-androstadienone-17

TABLE II

Method of Calculation of Results in DHA Colorimetric Assay

Determination	Mean L value	Estimated DHA content in aliquot	Factor	Total DHA content
				mg
No furfural blank	0 0000			
Reagent blank	0 0459			
10 γ DHA standard	*0 1257*			
30 " " "	0 2818			
50 " " "	0 4034			
Urine Extract 57-16 (total volume, 50 ml)				
0 4 ml 1 5 dilution				
Extract	0 3714			
Blank	0 0163			
Net	0 3551	42 2	625	26 4
0 2 ml 1 5 dilution				
Extract	0 2231			
Blank	0 0078			
Net	0 2153	21 5	1250	26 8
Mean value for Extract 57-16				26 6

Results

Table III demonstrates the reliability of the colorimetric method when it is applied to "unknown" solutions of DHA, to mixtures of DHA and

androsterone, and to a crude urine extract with and without added DHA It is apparent from these results that DHA can be assayed in the presence of a non-chromogenic substance and that added DHA can be quantitatively

TABLE III

Application of Method to Unknown Solutions

Solution No	DHA added	Androsterone added	Urine Extract 409 added	DHA found	Per cent recovery
	γ	γ	*ml*	γ	
1	32 5			33 0	101 5
2	22 5			22 5	100
3	32 0			32 4	101 2
4	12 0			12 0	100
5	20 0	40		20 2	101
6	40 0	80		39 8	99 5
7	50 0	100		50 0	100
8	0		0 5	14 5	
9	10 0		0 5	24 5	100
10	0		1 0	29 0	
11	20 0		1 0	49 0	100

TABLE IV

Color Intensity from Application of Method to Pure Compounds

	per cent
Dehydroisoandrosterone	100
" acetate	100
Potassium dehydroisoandrosterone sulfate	105
Δ^5-3(β)-Chloroandrostenone 17	102
$\Delta^{3,5}$ Androstadienone-17	80
Δ^5 Pregnenol-3(β)-one-20	78
Δ^5-Pregnenediol-3(β)21 one 20,21-acetate	>100
Δ^5-17-Ethinylandrostenediol 3(β),17(β)	75
Δ^5-17-Methylandrostenediol 3(β),17(β)	25
Δ^5 Androstenediol-3(β),17(α)	25
Δ^5-Androstenediol-3(β),17(β)	25
Δ^5-Androstenetriol-3(β),16,17	75
Δ^5-Androstenol-3(β)	55
$\Delta^{3,5}$-Androstadienol-17-acetate	25
Δ^{16} Allopregnenediol-3(α),20(β)	25
Δ^5 Pregnenediol-3(β),20(β)	<20

measured in the presence of a relatively crude urine extract Since the results suggested that the colorimetric determination of DHA in urine extracts was possible, the specificity of the reaction was investigated Table IV lists a series of neutral steroids tested which gave positive results when the color reaction was applied under the conditions previously

TABLE V

Pure Compounds Tested with Negative Results at 660 mμ

C_{19}-saturated	Androstanol-3(α)-one-17 (androsterone)
	Etiocholanol-3(α)-one-17
	Androstanol-3(β)-one-17 (isoandrosterone)
	Etiocholanol-3(α)-dione-11,17
	Androstanediol-3(α),11(β)-one-17
	Androstanol-5-trione-3,6,17
	Androstanedione-3 17
	Etiocholanetrione-3,11,17
	Androstanediol-3(α),17(α)
	Androstanetriol 2,3 17
C_{19}-unsaturated	$\Delta^{2\,(or\ 3)}$-Androstenone-17
	Δ^{9}-Androstenol-3(α)-one-17
	Δ^{9} Etiocholenol 3(α) one-17
	Δ^{4}-Androstenol 17(α)-one-3 (testosterone)
	Δ^{4}-Androstenedione-3,17
	Δ^{5}-Androstenedione 3 17
	Δ^{4}-Androstenetrione-3 11,17 (adrenosterone)
	Δ^{4}-Androstenetriol 3(β),4 17(α)
	Δ^{4}-17 Methylandrostenol-17(β)-one-3 (methyl testosterone)
C_{21}-saturated	Pregnanol 3(α)-one-20
	Pregnanol-20(α) one 3
	Allopregnanol 3(α)-one-20
	Allopregnanol-3(β) one-20
	Pregnanedione 3,20
	Allopregnanedione 3 20
	Pregnanetrione 3,11 20
	Allopregnanetrione-3 6 20
	Allopregnanediol 3(β) 6(α) one-20
	Pregnanediol 3 4 one 20 diacetate
	Pregnanediol-3(α),17(α) one 20
C_{21} unsaturated	Δ^{4} Pregnenedione-3,20 (progesterone)
	Δ^{4}-Pregnenol 17(α) dione 3,20
	Δ^{4}-17-Ethinylandrostenone 3
	$\Delta^{5,16}$ Pregnadiendiol-3 4 one 20 diacetate
	Δ^{16}-Allopregnenediol 3(β),6(β)-one-20
	Δ^{16} Pregnenedione 3 20
	17 Isopropylidenetiocholanol-3(β)
	Δ^{4} Pregnenediol-11(β),21-dione-3,20 (corticosterone)
	Δ^{4}-Pregnenediol 17(α),21-trione-3,11,20 (Kendall's Compound E)
	Δ^{4} Pregnenol 21 dione-3,20 (desoxycorticosterone)
Phenols	Estrone
	α Estradiol
	Estriol

specified In Table V is listed a series of neutral steroids tested which gave less than 5 per cent of the color produced by an equivalent weight of DHA, and are therefore considered to be negative

It is to be noted from the results presented in Table IV that, with the exception of DHA and its simple derivatives, only four *ketonic* steroids gave a positive color test Two of these, namely Δ^5-3(β)-chloroandrostenone-17 and $\Delta^{3,5}$-androstadienone-17, are known to occur in urine extracts, and are generally believed to be transformation products derived from DHA in the course of the hydrolytic procedure utilized to free the steroids from their conjugates The determination of these compounds as DHA would not be erroneous, therefore, and could be advantageous since the formation of artifacts is difficult to control or eliminate The other two ketonic steroids, pregnenolone and Δ^5-3(β)-hydroxy-21-acetoxy-20-ketopregnene, have not as yet been demonstrated in urine, although pregnenolone has been found in pig testis by Prelog and coworkers (6) 3(β)-21-Dihydroxy-20-ketopregnene has not as yet been isolated from natural sources Since the remainder of the compounds in Table III are all non-ketonic, they should not interfere with the determination of DHA by the colorimetric procedure, provided that the extract has first been separated into ketonic and non-ketonic fractions by a suitable procedure

Since the relation between color production and concentration of DHA is not strictly lineal in this procedure, and in the application to urine extracts Δ^5-3(β)-chloroandrostenone-17 and $\Delta^{3,5}$-androstadienone-17 would be determined as DHA, the proportionality between color and concentration of these substances must be established It can be seen from Fig 1 that, while DHA and the chloroketone give identical color intensity over the range of concentrations investigated, the slope of the curve relating color and concentration for $\Delta^{3,5}$-androstadienone-17 departs markedly from that of DHA While at 10 γ the agreement with DHA is excellent, with increasing concentration of dienone a relatively lower color intensity is developed, a result very probably to be ascribed to the greater destruction of the doubly unsaturated compound under the drastic conditions of the reaction In order to test whether low concentrations of $\Delta^{3,5}$-androstadienone-17 could be accurately assayed in the presence of DHA and 3(β)-chloroandrostenone-17, a mixture of these three substances in equal proportions was prepared and assayed at 10 and 30 γ levels In both instances the calculated value was obtained for the mixture, and it is therefore apparent that unless $\Delta^{3,5}$-androstadienone constitutes more than one-third of the chromogenic steroids in the color reaction no serious deviation from the true value is encountered For greatest accuracy with higher concentrations of the dienone the assay must be conducted with amounts of about 10 γ A distinction between DHA and the chloroketone or the dienone can be obtained by separation of the ketonic fraction into α and β subfractions by precipitation with digitonin Of the three compounds only DHA is precipitable with digitonin, and therefore the two transformation products will be found in the α-ketonic fraction

Table VI illustrates the application of the colorimetric determination of DHA to urine extracts The fractions studied were kindly supplied to us by Dr Richard Landau of the Department of Medicine, University of Chicago, and Dr Konrad Dobriner of the Sloan-Kettering Institute, New York The extracts were selected primarily on the basis of availability and are therefore not to be considered characteristic of either the normal or the diseased state The values obtained do serve to indicate, however, the fact that chromogenic compounds of the type of DHA and its transformation products constitute an appreciable percentage of the ketosteroid

TABLE VI

Colorimetric Estimation of Dehydroisoandrosterone in Ketonic Fraction of Urine Extracts

Ketosteroids*			Dehydroisoandrosterone equivalents		
Total ketonic fraction	α Ketonic fraction	β Ketonic fraction	Total ketonic fraction	α Ketonic fraction	β Ketonic fraction
mg	mg	mg	mg	mg	mg
8 5			2 2		
7 6			1 1		
2 8			0 3		
0 44			0 07		
0 23			0 05		
0 21			0 04		
	5 9	0 1		0 24	0 03
	7 0	1 2		0 93	0 27
	8 8	0 28		0 56	0
	5 1	0 16		0 35	0 05
	35 0†	30 0		10 1	26 2

* Measured by the Holtorff and Koch (7) modification of the Zimmermann (8) reaction

† Patient with an adrenal tumor

fraction of urine It is noteworthy that in each instance in which α- and β-ketonic fractions from the same urine have been assayed, a considerably higher value is obtained in the α-ketonic fraction than in the β-ketonic fraction The exception is a patient with an adrenal tumor in which the excretion of DHA was at a very high level Even under these circumstances approximately 40 per cent of the total chromogen was obtained in the α-ketonic fraction In view of the specificity of the color reaction it may therefore be suggested that the procedures used in the preparation of these urine extracts have resulted in the conversion of a considerable portion of DHA to the chloroketone and the dienone

DISCUSSION

From the results obtained with neutral steroids tested and reported here, as well as with the acidic and neutral compounds investigated by Kerr

and Hoehn (2), the structural requirements for a positive Pettenkofer test can be generalized Unsaturation in Ring B, or a group which can give rise to unsaturation in Ring B, and a hydroxyl group or double bond in Ring A are both essential for the reaction Of the compounds tested, the simplest substance conforming with these requirements is Δ^5-3(β)-hydroxy-androstene, which is approximately one-half as chromogenic as DHA All other substances giving a positive reaction possess an additional functional group in the molecule Thus cholesterol, which differs from 3(β)-hydroxy-Δ^5-androstene only by the aliphatic side chain at C-17, is non-chromogenic, while 3(β)-hydroxy-Δ^5-lithocholenic acid gave a positive test (2) The intensity of color is thus markedly influenced by the other functional groups in the molecule and an approximation of this effect may be made A carboxyl group at C-24 is equivalent to a ketone group at C-17, a ketol at C-20,C-21 increases the chromogenic effect, a ketone at C-20 is less effective than a C-17 ketone, and replacement of a ketone by a hydroxyl group at the same position decreases the color intensity, two hydroxyl groups in Ring D are approximately equivalent to a C-20 ketone in the color reaction It should be noted that Δ^{16}-3(α),20(β)-dihydroxyallopregnene gave a positive test, whereas $\Delta^{5,16}$-3,4-dihydroxy-20-ketopregnadiene was negative The color reaction, therefore, while not completely specific, is qualitatively indicative of a C-3 hydroxyl together with actual or potential unsaturation in Ring B and oxidation elsewhere in the molecule

The application of this color test to the non-ketonic fraction of urinary extracts should prove of value, since isolation experiments have shown that chromogenic compounds such as Δ^5-3,17-dihydroxyandrostene and Δ^5-3,16,17-trihydroxyandrostene are present in this fraction . Further investigation of the specificity of the reaction would be desirable before any considerable reliance could be placed on the method as a means of identification of individual non-ketonic steroids, but extension to this relatively unexplored fraction should not be overlooked

We wish to express our appreciation for the advice and encouragement received from the late Professor F C Koch throughout the course of this investigation We also acknowledge the technical assistance of Mr Arthur A Wykes, and thank the Wm S Merrell Company, Cincinnati, and Dr Robert S Shelton, Vice President, for permission to use their laboratory facilities during the summer of 1943 Among the investigators to whom we are indebted for samples of crystalline steroids are Dr Wayne Cole, Dr Lewis Engel, Dr Seymour Lieberman, Dr H L Mason, Dr A D Odell, Dr R E Marker, and Dr C R Scholz

SUMMARY

1 A colorimetric analytical method for dehydroisoandrosterone (DHA),

which is applicable to urine extracts as well as to pure solutions, has been described It is based on the color produced when acetic acid solutions containing 10 to 50 γ of the steroid are treated at 67° with furfural and sulfuric acid

2 The specificity of the method has been investigated with 56 different crystalline neutral steroids Forty of the compounds gave no significant color

3 The sixteen compounds which gave positive tests may be divided as follows (*a*) DHA and simple derivatives including the transformation products Δ^5-3(β)-chloroandrostenone-17 and $\Delta^{3,5}$-androstadienone-17, derived from DHA during acid hydrolysis of urine, (*b*) two other neutral ketones, pregnenolone and 21-acetoxy pregnenolone, which have not as yet been shown to occur in urine, and (*c*) non-ketonic unsaturated steroids

4 In general the color test indicates the presence of unsaturation or potential unsaturation in Ring B and a hydroxyl group or double bond in Ring A

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THE EFFECT OF pH UPON THE TOXICITY OF IODOACETIC ACID TO YEAST CELLS*

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In investigating the respiratory metabolism of cells whose growth has been inhibited by the use of various agents, it is often advantageous for the action of the inhibitor to be irreversible Following an exposure to an inhibitory agent of this type, the cells may be freed from the medium containing the inhibitor, thus permitting an investigation of the altered metabolism under a variety of environmental conditions

The fact that monoiodoacetic acid is a potent inhibitor of fermentation was demonstrated many years ago by Lundsgaard (1) That this inhibition was dependent upon pH was noted by several workers Ehrenfest (2) reported that yeast fermentation was totally inhibited by 3.6×10^{-4} M at pH 4 6 but that this inhibition gradually decreased as the medium was made more alkaline, until at a pH of 7 0 no inhibition was apparent She concluded that changes in cell permeability were responsible for these results and produced evidence purporting to support this conclusion

Following these early observations iodoacetic acid was widely used as a tool for investigating the mechanism of carbohydrate metabolism in a variety of cells and tissue preparations In spite of this work there resulted little unanimity of opinion as to the specific mode of action of the compound Both Dickens (3) and Lohmann (4) showed that iodoacetic acid inhibited glyoxalase in liver slices and, from the fact that reduced glutathione restored the activity of their inhibited preparations, concluded that —SH groups were involved in the inhibition This work was carried on at a pH of 7 4

With the exception of a paper by Cayrol (5) little appears to have been reported regarding the toxicity of iodoacetic acid This author observed that in acid media respiration, fermentation, and cell division were inhibited, while at neutrality only fermentation was effected by the same concentration of inhibitor

Recently Kinsey and Grant (6) classified iodoacetic acid as a "reversible" inhibitor because the depression of growth and respiration observed while the drug was in contact with the cells disappeared upon resuspending the cells in fresh medium The pH of the medium here was 5 5

Work with iodoacetic acid has been in progress in this laboratory for a

* This investigation was supported by funds from the National Cancer Institute of Canada

year, and under the experimental conditions employed the effect of the inhibitor was always irreversible. Since exposures of the yeast suspensions to the poison were always made at a pH of 4.5 and under these conditions the results were at a variance with those reported at slightly more alkaline conditions (pH 5.5), it was decided to investigate in detail the question of whether acidity of the medium was responsible for these inconsistencies. These studies emphasize the fact that pH has a marked effect both upon the dissociation of iodoacetic acid and upon the toxicity of the inhibitor to yeast cells, especially over the range, pH 3.5 to 5.5. Moreover, the toxicity appears to be directly proportional to the concentration of the undissociated iodoacetic acid molecules.

Materials and Methods

Method A.—Cells of *Saccharomyces cerevisiae* originally obtained from a single cell isolation were grown for 18 hours on plates of Miller (7) agar medium incubated at 20°. After harvesting, the cells were washed twice by centrifugation with phosphate buffer (0.05 M KH_2PO_4) and were finally made up as a 1 per cent suspension in phosphate buffer. Aliquots of this suspension were then centrifuged and the packed cells resuspended in phosphate buffer adjusted to pH values of 3.5, 4.0, 4.5, 5.0, and 5.5. 5 ml. of each suspension were placed in each of five L-shaped test-tubes immersed in a constant temperature bath at 20°. 3 ml. of phosphate buffer at the corresponding pH were added plus 1 ml. of 10 per cent solution of glucose in phosphate buffer adjusted to the corresponding pH. This mixture was shaken for ½ hour, at which time 1 ml. of 10^{-2} M iodoacetic acid (pH 4.5) was added to each suspension. A sixth aliquot to which no iodoacetic acid was added served as a control. After ½ hour each tube was sampled for viable cells, the procedure being that of serial dilution. Sampling, diluting, and the final plating out into the Miller gelatin medium were all carried out in duplicate.

In two experiments in this series, the KH_2PO_4 buffer was replaced by the McIlvaine citric acid-phosphate buffer after it had been established that this procedure did not alter the characteristics of the respiratory metabolism. Identical results were obtained in both cases.

Results

Viable cell counts carried out according to the above procedure varied from 0 to 100 per cent (per cent of the control colony count) as the pH was increased from 3.5 to 5.5. The actual figures are recorded in Table I, from which it is immediately apparent that the greatest toxicity is produced in the most acid environment.

The possibility that these results might be due to varying degrees of

dissociation of the iodoacetic acid was then considered Accordingly, the percentage dissociation of the acid at various points throughout the range covered in the above experiments was calculated, and, since toxicity decreases with increasing dissociation, the per cent *undissociated acid* was plotted against pH Such a plot is indicated in Fig 1

When viability is plotted against concentration of undissociated acid, as has been done in Fig 2, one obtains a fair approximation to a straight line This may be taken as an indication that the irreversible toxic action of iodoacetic acid is directly proportional to the concentration of the undissociated molecule Confirmatory evidence for this conclusion was sought by an entirely different procedure based upon the following reasoning

TABLE I

Influence of pH upon Toxicity of Iodoacetic Acid

The amount of iodoacetic acid added in all cases was 1 ml of 10^{-2} M to 9 ml of yeast suspension The per cent viability represents the per cent of the control (untreated) colony count Undissociated acid concentrations were computed from Fig 1

pH*	Per cent viability	Undissociated acid, $\times 10^{-4}$ M
3 5	0	8 0
3 7	0	7 0
3 9	0	6 0
4 1	5	5 0
4 35	15	3 8
4 5	40	3 1
4 85	81	1 6
5 0	100	1 2
5 45	90	0 4
5 5	100	0 4

* pH measurements taken immediately prior to addition of iodoacetic acid to yeast suspension

If the concentration of the undissociated acid is the factor responsible for the toxicity of the drug, then one should be able to expose the cells to varying concentrations of iodoacetic acid under conditions of constant pH and observe much the same phenomenon Moreover, since the degree of dissociation at any pH is known, the viable cell counts from such an experiment, when plotted against the concentration of undissociated acid (rather than against concentration of the acid added), should fall along the same line as that shown in Fig 2 A series of such experiments was carried out as indicated below

Method B—The harvested, washed cells were made up as a 1 per cent suspension in phosphate buffer, pH 4 5 Each L tube received 5 ml of

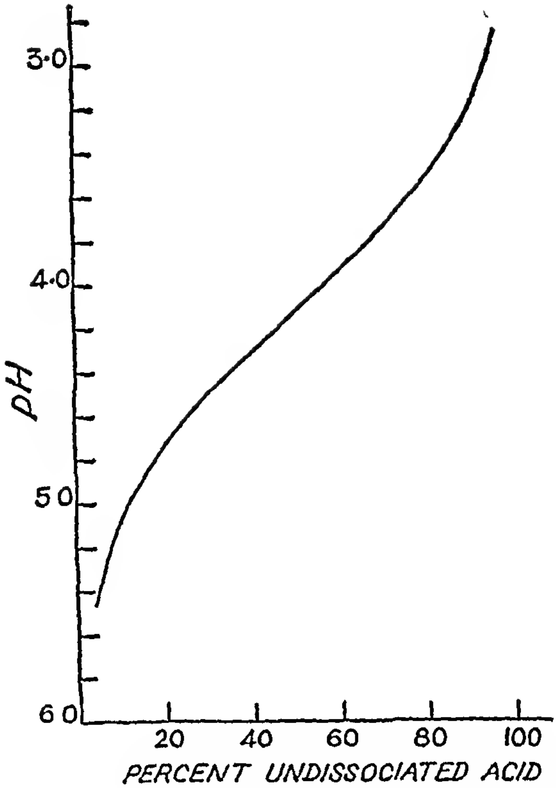

FIG 1 The dissociation curve for iodoacetic acid calculated from $K_D = 7\ 5 \times 10^{-4}$

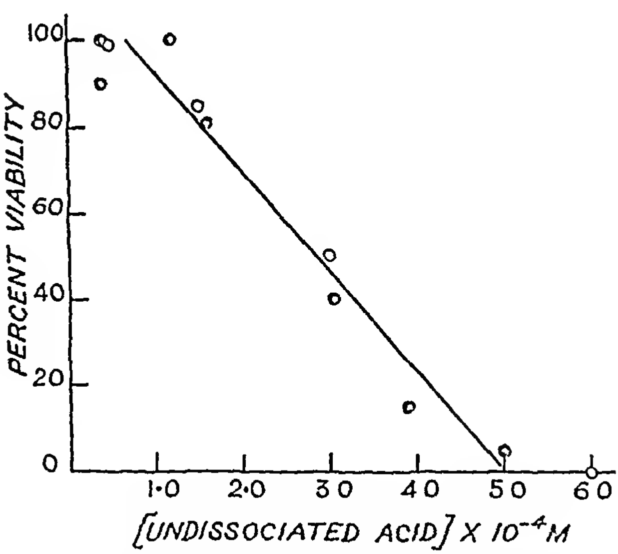

FIG 2 Viability of yeast cells as a function of the concentration of undissociated iodoacetic acid **o**, the result from experiments in which the undissociated acid concentration was varied by changing the pH of the medium (Method A) O, the result from experiments carried out under conditions of constant pH the undissociated acid concentration being varied by changing the concentration of the inhibitor that was added to the yeast suspension (Method B)

yeast suspension, various amounts of phosphate buffer, and 1 ml of 10 per cent glucose solution After the ½ hour equilibration period, various quantities of 10^{-2} M iodoacetic acid (to make the total volume of each tube to 10 ml) were added and the whole mixture shaken for ½ hour prior to sampling for viable cells In some experiments in this series the KH_2PO_4 was replaced by the citric acid-phosphate buffer (pH 4 5) with no change in results The final concentration of iodoacetic acid in this series ranged from 2×10^{-4} M to 2×10^{-3} M Since, at a pH of 4 5, 30 per cent of the acid exists in the undissociated form, each of the concentrations of acid used was multiplied by 0 3 and the resulting values from the four experiments together with corresponding viabilities were plotted in Fig 2

DISCUSSION

It will be noted that the points in Fig 2 show a certain amount of scatter about the line intended to represent their average This situation is largely due to the difficulty in maintaining the pH at any given value in this acid range

The maintenance of constant pH in a suspension of yeast cells that are actively metabolizing glucose presents a difficult problem, especially if one seeks to use a buffer which does not modify the metabolic characteristics of the cells Potassium dihydrogen phosphate is a poor buffer over the range covered in the above experiments, and its use necessitated frequent pH determinations For this reason all of the experiments were repeated with the McIlvaine citric acid-sodium phosphate buffer, which was found to maintain the pH of the medium at its original value over reasonably long periods of time

The interpretation of the data presented in Fig 2 is fairly obvious, although it is somewhat surprising to find that the undissociated molecule rather than the iodoacetyl ion is the species responsible for the irreversible toxicity

Preliminary measurements of the respiratory characteristics of the suspensions treated even with low concentrations of iodoacetic acid show almost a complete absence of both aerobic respiration (oxygen consumption and CO_2 production) and anaerobic fermentation This association of irreversible cellular damage with cessation of respiratory activity is interesting when compared to the situation obtaining under more alkaline conditions In those experiments in which an exposure to iodoacetic acid was made at a pH of 5 5, all the cells developed normally and showed very little change in respiratory activity Nevertheless, under these and more alkaline conditions, when the iodoacetyl ion predominates, a well defined inhibition of respiration has been noted as long as the toxic ion is present in the medium Here, however, there appears to be a selectivity of action asso-

ciated with the ion, since CO_2 production is depressed but oxygen consumption frequently remains unaltered. This would seem to point to a difference in specificity of action between the ion and the molecule, although one must also consider the possibility that irreversible cellular damage (obtained under acid conditions) may in itself lead to cessation of all respiratory activity. Certainly the above experiments indicate that the iodoacetic acid molecule has a much greater affinity for the cell than does the iodoacetyl ion.

SUMMARY

As a result of experiments performed under conditions of varying pH and of varying iodoacetic acid concentration it has been demonstrated that the irreversible toxic action of the inhibitor toward yeast cells is proportional to the concentration of undissociated iodoacetic acid. At pH values lower than 5.0 the action of the inhibitor is irreversible, while in media more alkaline than 5.0 the action is reversible.

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STUDIES ON AMINO ACID EXCRETION IN MAN

II AMINO ACIDS IN FECES*

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The fecal nitrogen of normal man remains relatively unchanged despite considerable variation in protein nitrogen intake, provided that the dried weight of food is kept constant (1) Whether or not the fecal amino acids maintain a similar constancy despite variation in the amino acid content of the diet has not been adequately investigated heretofore

The purpose of this paper is to report the data obtained by microbiological measurement of eight amino acids in the food and in the feces of two healthy, adult men fed a variety of diets The preparation of feces samples for amino acid analysis by the microbiological technique also is described

Analyses were performed on acid-hydrolyzed samples, and therefore the total amounts of the amino acids methionine, lysine, arginine, histidine, leucine, isoleucine, valine, and threonine were measured The results indicate that, despite great differences in the amounts ingested, the quantity of individual amino acids as well as the total nitrogen in the feces remains relatively constant

EXPERIMENTAL

As described previously (2), the subjects were two healthy young men hospitalized for the purposes of this investigation in the metabolism section of the Albert Merritt Billings Hospital They were permitted normal activity Each diet was studied for two consecutive 6 day periods, following a 3 day interval which permitted adjustment of the subjects to the change in food Though the intake of protein was varied, the caloric intake and the weight of dried matter were kept as uniform as possible throughout the study by adjustment of the carbohydrate content of the diet Sufficient amounts of carbohydrate, fat, and vitamin supplements were administered to make the diets nutritionally complete The preparation and

* A portion of this study was presented before the Division of Biological Chemistry at the Chicago meeting of the American Chemical Society, April, 1948

This study was supported in part by grants from the Evaporated Milk Association and the American Dairy Association

analysis of the food and the microbiological procedure utilized in this study have been described in a previous publication (2)

Except in Periods 9 and 10, in which crystalline methionine was added to the diet, the amount of individual amino acids ingested was varied by changing either the quantity or type of protein in the food With the exception of methionine, no attempt was made to study the effects of added individual amino acids upon the excretion in feces of the other amino acids

Collection and Preparation of Feces Samples for Analysis—Feces were collected into a large, weighed, covered glass mixing bowl and stored in the refrigerator for each 6 day period There was no obvious decomposition of the feces through growth of bacteria or mold Each 6 day collection was marked by 0 3 gm of carmine administered to the subjects just prior to the first meal of the period and at the end of exactly 6 days, the feces between the two markers were saved for analysis

The total collection of feces for each period was weighed and homogenized with distilled water in a Waring blendor Measured aliquots were then taken for total nitrogen and for amino acid analysis Total nitrogen was determined by the semimicro-Kjeldahl procedure with selenium dioxide and cupric sulfate as the digestion catalysts

For amino acid analysis, 2 5 to 3 gm of the homogenized sample were weighed accurately into a conical beaker and autoclaved with 20 ml of 4 N hydrochloric acid for 10 hours at 120° The samples were then boiled 10 minutes with 250 mg of norit A, filtered by suction, adjusted to pH 6 8, and made up to a volume of 1 liter All samples were hydrolyzed in duplicate and each hydrolysate was analyzed at three different levels of concentration

The norit A treatment is important in the preparation of the samples because it removes certain unidentified substances in feces which stimulate the growth of the assay organisms and cause apparently excessive recoveries of added amino acids Recovery of pure amino acids added to normal human feces before hydrolysis ranged from 93 to 108 per cent

RESULTS AND DISCUSSION

The data for the eight amino acids are presented in Table I Since there were no significant differences between the results for each subject, the values for both in each period were averaged together The fecal nitrogen remained relatively constant throughout the study

The intake of histidine per 6 day period ranged from 6 9 to 13 8 gm The variation in the intake of the remaining seven amino acids was greater, being highest for lysine, 13 2 to 31 7 gm per period Nevertheless, the total output of each amino acid in the feces per 6 day period did not vary more than 0 55 gm, plus or minus, from the mean output for the particular

amino acid The values for methionine in the feces never varied more than 0.2 gm from the mean, even when 6.0 gm of DL-methionine were added to a diet adequate to maintain the subjects in positive nitrogen balance

TABLE I

Microbiologically Available Amino Acids in Feces of Two Adult Men Fed Various Diets

Each value represents the averaged result for the two subjects studied for a 6 day period

Period No	Methionine		Lysine		Arginine		Histidine	
	Ingested	In feces	Ingested	In feces	Ingested	In feces	Ingested	In feces
	gm	gm	gm	gm	gm	gm	gm	gm
1	11.5	0.8	31.7	2.9	26.0	1.9	13.8	0.7
2	11.5	0.6	31.7	2.6	26.0	2.1	13.8	0.8
3	6.4	0.7	13.2	2.1	13.3	1.3	6.9	0.6
4	6.4	0.8	13.2	2.6	13.3	1.6	6.9	0.7
5	11.0	0.7	30.8	2.3	24.7	1.6	12.7	0.7
6	11.0	0.7	30.8	2.4	24.7	1.4	12.7	0.7
7	8.8	0.5	23.8	1.9	23.6	1.2	11.9	0.7
8	8.8	0.7	23.8	2.2	23.6	1.5	11.9	0.7
9*	14.8	0.9	23.8	2.5	23.6	1.7	11.9	0.9
10*	14.8	0.7	23.8	2.3	23.6	1.6	11.9	0.7

Period No	Leucine		Isoleucine		Valine		Threonine	
	Ingested	In feces	Ingested	In feces	Ingested	In feces	Ingested	In feces
1	39.0	2.9	28.4	2.3	27.5	2.6	19.8	2.2
2	39.0	2.5	28.4	2.0	27.5	2.4	19.8	1.9
3	24.7	2.2	17.1	1.6	16.9	1.9	11.5	1.5
4	24.7	2.5	17.1	1.8	16.9	2.0	11.5	1.4
5	37.0	1.8	27.3	1.5	24.5	1.5	18.5	1.6
6	37.0	1.8	27.3	1.7	24.5	1.8	18.5	1.7
7	34.7	2.0	22.2	1.6	23.0	1.5	17.2	1.4
8	34.7	2.4	22.2	1.4	23.0	1.6	17.2	1.6
9*	34.7	2.8	22.2	2.2	23.0	2.2	17.2	1.7
10*	34.7	2.4	22.2	1.7	23.0	1.9	17.2	1.7

* 6 gm of DL methionine added per period, supplementing the diet fed during Periods 7 and 8

To study the other extreme, one of the subjects was later placed on a diet, the major protein of which was supplied by a peptone solution treated with 3 per cent hydrogen peroxide to destroy some of its methionine This diet was followed by a regimen in which the peptone was treated with 30 per cent hydrogen peroxide In both instances the daily diet was supplemented by 1.25 gm each of DL-tryptophan, L-tyrosine, and L-cystine to compensate for the destruction of these amino acids by the peroxide The

total quantity of methionine ingested was only 2.3 gm. in the first 6 day period and 0.9 gm. in the latter. The amount of methionine excreted in the feces in each period was 0.9 and 0.6 gm. respectively, values within 0.2 of the mean established on diets containing adequate quantities of methionine. Although the hydrogen peroxide-treated peptone is relatively low also in histidine and leucine, the quantities of these amino acids in the feces likewise did not decrease during the ingestion of the peptone diet.

It is apparent, therefore, that the quantity of amino acids excreted in the feces remains comparatively constant despite relatively large changes in the individual amino acid content of the diet. Also, analysis of the dilute acid extract of feces, deproteinized by tungstic acid precipitation, reveals minute, if any, amino acid activity microbiologically, and hydrolysis of the protein-free extract does not increase this activity. Therefore, practically none of the fecal amino acids can be considered as free, polypeptide, or conjugated amino acids.

No conclusions can be made concerning the direct source of fecal amino acids on the basis of the data obtained in this study. However, the results indicate that the nitrogen represented by any of the eight amino acids studied maintains a fairly constant proportion to total fecal nitrogen. This finding is in agreement with the commonly accepted thesis that the greatest portion of fecal nitrogen is in the form of bacterial protein and that the intestinal bacteria produce protein of constant composition. In addition, if the intestinal bacteria derive nutrition from unabsorbed proteins and amino acids, variation in the amino acid content of the diet, although causing no change in the composition of bacterial protein, would, however, produce a variation in the total quantity of protein in the feces. The relatively constant excretion in the feces of the eight amino acids studied indicates, therefore, that the fecal amino acids are not unabsorbed residues of the diet. Presumably, they represent part of a constant supply of protein, probably digestive enzymes, secreted into the lumen of the gastrointestinal tract and excreted as such or converted into bacterial protein before elimination from the body.

SUMMARY

1. The total quantity of each of the amino acids methionine, lysine, arginine, histidine, leucine, isoleucine, valine, and threonine was measured microbiologically in the food and feces of two normal adult men.

2. The special preparation of feces samples for the amino acid analysis by the microbiological technique is described.

3. The results indicate that, despite great differences in the amounts fed, the total quantity of individual amino acids excreted in the feces remains comparatively constant.

4 It is suggested that these fecal amino acids represent amino acids secreted into the lumen of the gastrointestinal tract as components of digestive enzymes, excreted as such or converted into bacterial protein before elimination from the body

The authors wish to express their gratitude to Miss Blanche Parish, R N, for supervising the collections and care of the subjects, to Miss Minnie Brandt for composing and preparing the diets, and to the subjects, Mr John Doull and Mr Richard Herz, for their cooperation

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STUDIES ON THE CYCLOPHORASE SYSTEM

II THE COMPLETE OXIDATION OF FATTY ACIDS

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In the first paper of this series (1) a detailed description was given of the method of preparation and the properties of the cyclophorase system from rabbit kidney and liver which catalyzed the complete oxidation of pyruvic acid to carbon dioxide and water by way of the citric acid cycle The present communication deals with the complete oxidation of fatty acids to carbon dioxide and water as catalyzed by the same cyclophorase-containing preparations from kidney and liver

Leloir and Muñoz (2–4) were the first to achieve β oxidation of fatty acids in cell-free preparations from animal tissues If we consider the extreme instability of the system from rat and guinea pig liver with which they worked, it is indeed remarkable that they should have discovered essentially all the important requirements for activity of the enzyme system They established the necessity for the following four components (*a*) magnesium ions, (*b*) phosphate ions, (*c*) adenylic acid or adenosine triphosphate, and (*d*) cytochrome *c* They recognized that the oxidation of fatty acids had to be primed or sparked, as it were, by the simultaneous oxidation of one of the intermediates in the citric acid cycle They observed that coincident with the oxidation of fatty acids inorganic phosphate was taken up while a substance answering the description of phosphopyruvic acid accumulated Under one set of conditions, butyric acid was oxidized only as far as acetoacetic acid, while under other conditions, or even without a change in the conditions, butyric acid was oxidized beyond the stage of acetoacetic acid Leloir and Muñoz found that fatty acids from C_4 to C_8 were oxidized most rapidly in their liver mince

More recently the problem has been taken up by Lehninger (5–10), who has described a malonate-insensitive system in liver which catalyzes the oxidation of octanoate and other fatty acids quantitatively to acetoacetic

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acid The malonate insensitivity of Lehninger's system contrasts with the malonate sensitivity of the Leloir and Muñoz system Lehninger has also observed that the oxidation of fatty acids can be sparked by the cooxidation of α-ketoglutarate, but he has claimed in addition that adenosine triphosphate in large excess can replace the cooxidation system The necessity for adenosine triphosphate led Lehninger to the view that the fatty acids were phosphorylated enzymatically with the formation of acyl phosphates

Results

Oxidation of Fatty Acids by Kidney

Components of Fatty Acid Oxidation System—The kidney enzyme system prepared as described under "Experimental" will oxidize fatty acids when fortified with adenosine triphosphate (or adenosine monophosphate), magnesium ions, and inorganic phosphate In numerous studies with acetic, butyric, β-hydroxybutyric, crotonic, vinylacetic, and β-ketocaproic acids, it was observed that the rate of fatty acid oxidation may be strikingly reduced in the absence of any one of these additions The magnitude of these effects is highly variable With a very fresh enzyme preparation they may be absent or slight, or appear only rather late in the experiment However, with a preparation even a few hours old they are usually apparent at the very beginning of the experiment, and become more marked as the experiment proceeds (Fig 1)

Rather early in the present investigation it was noted that the rates of oxidation of various fatty acids, in the presence of adenosine triphosphate, magnesium, and phosphate, may frequently be increased significantly by adding to the system a small amount (0 3 cc) of a 1 per cent aqueous extract of an acetone powder of pig heart (Fig 1) Under the circumstances, this heart extract was included in the great majority of the experiments here reported In the following paper of this series, Knox *et al* (11) have presented evidence that the addition of the extract is equivalent to adding a small amount of the sparker or primer, *i e*, a substance which in catalytic amount potentiates the oxidation of a fatty acid

Oxidation of Fatty Acids by Kidney Preparations—Under the experimental conditions obtaining for Table I, kidney enzyme preparations have been found to oxidize rapidly (*a*) all of the saturated straight chain monocarboxylic fatty acids from acetic through *n*-tridecylic acid with the exception of propionic acid, (*b*) some substituted and unsaturated derivatives of these acids, and (*c*) the branched chain fatty acid, isocaproic acid

Oxygen uptake data from numerous manometric experiments are summarized in Table I, which is largely self-explanatory Substrates were always added to the main compartment prior to gassing and equilibration,

since oxidative activity is markedly reduced or abolished unless the enzyme is protected by substrate during equilibration Inasmuch as oxidation proceeds at an unknown and presumably variable rate during gassing and equilibration, the total oxygen uptake in such experiments is greater than the measured uptake by an amount which can only be guessed at by extrapolation As an aid to extrapolation, the rate of oxygen consumption during the first 5 minutes after closing the taps has been recorded in each case

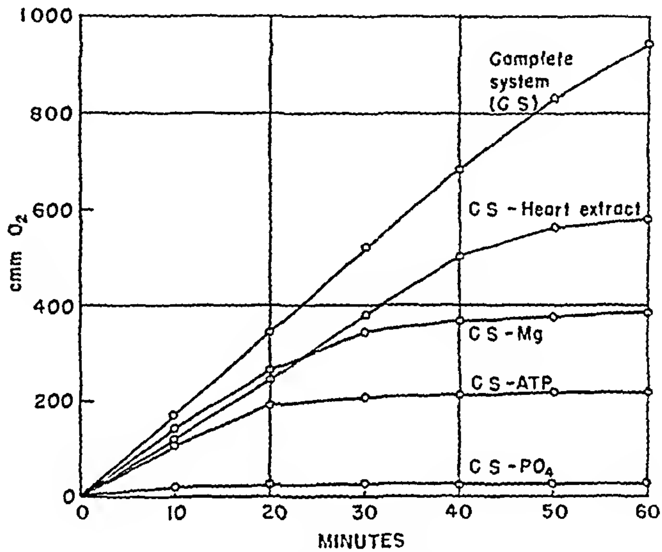

FIG 1 Oxidation of crotonic acid, component study The complete system included adenosine triphosphate, magnesium ions, phosphate buffer, heart extract, 30 micromoles of crotonic acid, and 1 cc of kidney enzyme (R_1K) used 5 hours after preparation Oxygen uptake in the control without added substrate was 50 c mm in 60 minutes

The data in Table I are consistent with the complete oxidation, to carbon dioxide and water, of all of the straight chain, saturated, unsubstituted fatty acids with an even number of carbon atoms from acetic through *n*-lauric acid, as well as of the following substituted or unsaturated derivatives of these acids crotonic, vinylacetic, and β-ketocaproic acids In some instances, *e g* acetic (10 micromoles) and vinylacetic acids, the recorded oxygen uptake is significantly in excess of theoretical requirements for complete oxidation, and the disparity becomes even more marked with extrapolation for the equilibration period These high values are interpreted as indicating the inadequacy of the correction for oxygen uptake in the control without added substrate This correction is based upon the

Table I
On Completeness of Fatty Acid Oxidation by Kidney System

In all the manometric experiments, the following additions were made 0.3 cc. of 0.01 M adenosine triphosphate, 0.2 cc. of 0.02 M magnesium sulfate, 0.5 cc. of 0.04 M phosphate buffer of pH 7.3, 0.3 cc. of heart extract, substrate, and 2 cc. of enzyme (R_3K added last) used immediately after preparation, alkali in the center well and 100 per cent oxygen in the gas phase, acids added in the form of neutralized sodium salts. The interval between the entry of the manometer into the bath at 38° and the closing of the stop-cock was timed and kept to 5 minutes. The values for recorded oxygen uptake have all been corrected for the oxygen uptake of the blank experiment without added substrate. All experiments were carried to completion, i.e. until oxygen uptake was at a standstill. The experiments lasted from 70 to 105 minutes in all but one instance (140 minutes). The total oxygen uptake in the controls without added substrate varied from 7 to 20 microatoms in the various experiments.

	Fatty acid	Oxygen uptake, *microatoms*			Theory, *microatoms oxygen*	
		Micromoles added	Recorded in 1st 5 min.	Total recorded	For complete oxidation to CO_2 and H_2O	For oxidation to propionic acid, CO_2 and H_2O
Even numbered	Acetic	10	7.0	47.7	40	
		15	6.0	54.5	60	
	Butyric	5	12.1	47.6	50	
		10	12.6	94.2	100	
		10	12.9	100.1	100	
	n-Caproic	5	11.5	74.6	80	
	Caprylic	5	11.7	99.3	110	
	Capric	1.25	8.8	33.4	35	
		2.50	8.4	66.4	70	
	Lauric	1	7.1	35.6	34	
Odd numbered	*n*-Valeric	5	7.5	30.0	65	30
		5	5.4	28.1	65	30
	Heptylic	2.5	13.8	24.8	47.5	30
		2.5	13.1	24.6	47.5	30
		2.5	12.3	20.7	47.5	30
		5.0	13.9	63.0	95	60
		5.0	16.0	58.5	95	60
		5.0	17.5	53.9	95	60
	Pelargonic	5	9.4	98.0	125	90
	Undecylic	1	10.1	19.6	31	24
		2	11.8	47.1	62	48
	$\Delta^{10,11}$-Undecylenic	1	8.4	24.4	30	24*
	Tridecylic	1	5.2	26.5	37	30
Branched chain	Isocaproic	5	5.6	33.0	80	30†
Derivatives	Crotonic	10	7.5	92.3	90	
	Vinylacetic	10	9.1	103.2	90	
	β-Ketocaproic	5	9.6	63.9	70	

* Theory is for oxidation to acrylic acid, CO_2 and H_2O.
† Theory is for oxidation to isobutyric acid, CO_2 and H_2O.

assumption that the endogenous substrates in the system are oxidized at the same rate in the presence as in the absence of added fatty acid It can be shown that this assumption is not valid in all cases and that a substance like pyruvic acid,[1] which is present in some enzyme preparations, remains unoxidized in the blank but undergoes complete oxidation in the experimental vessel coincident with the oxidation of fatty acids In such cases, the recorded oxygen uptake would exceed that attributable to oxidation of the fatty acid by an amount which would not be completely corrected for by that recorded in the blank without added substrate

As mentioned above, propionic acid is completely inactive in the kidney system The other saturated, straight chain, monocarboxylic fatty acids, from *n*-valeric acid through *n*-tridecylic acid, are all actively oxidized However, the recorded oxygen uptake in all cases is well below the theoretical requirement for complete oxidation to carbon dioxide and water The data are consistent with the oxidation of odd numbered fatty acids to propionic acid, carbon dioxide, and water, of isocaproic acid to isobutyric acid, carbon dioxide, and water, and of $\Delta^{10\ 11}$-undecylenic acid to acrylic acid, carbon dioxide, and water In Paper IV Atchley (13) has described the use of the counter-current distribution method for demonstrating the formation of non-oxidizable residues resulting from the oxidation of *n*-valeric acid and isocaproic acid

It will be noted in Table I that the amount (in micromoles) of substrate used in these experiments was never large, and that it was progressively reduced as the length of the carbon chain increased The reasons for this are twofold First, the activity of these enzyme preparations is regularly impaired and may be abolished by prolonged shaking at a temperature of 38° Secondly, some of the fatty acids, particularly the higher members of the series, may be readily oxidized at very low concentrations, but oxidized poorly or not at all at higher concentrations For example, *n*-capric acid was rapidly oxidized to completion at 1 25 and 2 5 micromoles per cup, but was completely inactive at 5 micromoles per cup, *n*-lauric acid was rapidly oxidized to completion at 1 micromole, was oxidized slowly and incompletely at 2 micromoles, and was entirely inactive at 5 micromoles per cup Even with *n*-valeric acid, this concentration effect may be readily apparent at 10 micromoles per cup and higher

A considerable number of fatty acids and their derivatives have been tested in manometric experiments, with negative results In the straight chain fatty acid series, formic, propionic, myristic, palmitic, stearic, and oleic acids were found not to be oxidized by the kidney system Iso-

[1] The estimation of pyruvic acid by the salicylaldehyde method of Straub (12) has disclosed that many preparations of the kidney system, even at the R_2 residue stage, form during the experiment as much as 1 micromole of pyruvic acid per cc of enzyme The origin of this pyruvic acid has not been ascertained

butyric, isovaleric, acrylic, dimethylacrylic, and $\Delta^{4,5}$-pentenoic acids were also inactive over a wide range of concentrations No α-substituted fatty acid proved active, *e g*, α-hydroxybutyric, α-ketobutyric, and α,β-dihydroxybutyric acids γ-Hydroxybutyric and 3,4-epoxybutyric acids were studied with particular interest as possible intermediates in the oxidation of butyric acid, in view of the rapid and complete oxidation of vinylacetic acid and succinic semialdehyde [2] Neither was found to show any activity The introduction of a methyl or phenyl group in the α or β position renders a fatty acid inactive, *e g*, 2-phenylbutyric and 3-methylcrotonic acids All the dicarboxylic acids tested were inactive, *e g*, glutaric, adipic, and azelaic acids

Oxidation of Postulated "Intermediates" of β Oxidation—Assuming that Knoop's classical theory of successive β oxidation (14) is applicable to the kidney system under investigation, we would anticipate that β-hydroxy, α,β-unsaturated, and β-keto acids corresponding to any oxidizable fatty acid should also be oxidized The data of Table II show that in all but one case[3] the postulated "intermediates"[4] of β oxidation are indeed oxidized What is also of considerable interest is that these "intermediates," whenever tested, have been found to be oxidized to the same end-products as the parent acids When the oxidation of a fatty acid involves merely the loss of a C_2 unit, and particularly when the product of oxidation is inhibitory, *e g* propionic acid from valeric acid, the β or $\Delta^{\alpha,\beta}$ derivatives of the parent acid are oxidized to completion only in the presence of a considerable amount of the sparker, even though the parent acid may get by on minimal amounts This discrepancy is not unexpected in view of the considerations brought up in foot-note 4 β-Hydroxylauric acid was found to be a profound inhibitor of the fatty

[2] Succinic semialdehyde is oxidized completely to carbon dioxide and water, as shown by the following experiment 22 8 micromoles were added at the beginning of the experiment The observed oxygen uptake due to succinic semialdehyde accounted for the complete oxidation of 13 4 micromoles, and there remained at the end of the experiment 8 8 micromoles (theory, 9 4 micromoles)

[3] According to the manometric data $\Delta^{10,11}$-undecylenic acid is oxidized down to acrylic acid $\Delta^{4,5}$-Pentenoic acid would be an "intermediate" in this degradation and, therefore, should be oxidized in the kidney system No success has attended many attempts to show that it undergoes oxidation in the kidney system The liver system can oxidize acrylic acid to carbon dioxide and water, and it is of interest that $\Delta^{4,5}$-pentenoic acid is readily oxidized in the liver system Its inactivity in the kidney system may well be due to inhibitory effects either of the acid itself or of its product of oxidation (acrylic acid)

[4] The term "intermediates" is placed in quotation marks since, unlike true intermediates, the substances listed in Table II are not oxidized as such by the enzyme system, but only after they have undergone a transformation brought about by the sparker In another communication, the nature of this transformation will be considered in some detail

acid oxidase even at concentrations less than 0 1 micromole per cc However, it was possible to demonstrate that it was oxidized to within 75 per cent of completion when highly active preparations of the enzyme were

TABLE II

Completeness of Oxidation of β-Hydroxy, β-Keto, and α,β-Unsaturated Fatty Acids by Kidney System

Details as in Table I, except that heart extract was omitted and 5 micromoles of α-ketoglutaric acid were used as the sparker

Fatty acid derivative	Micro moles added	Micro-moles utilized*	Microatoms oxygen absorbed (blank subtracted)	Theory for† complete oxidation to CO_2 and H_2O	Theory for oxidation to propionic or isobutyric acid and CO_2 and H_2O
				microatoms O_2	*microatoms O_2*
dl-β-Hydroxybutyric	2 0		20 4	18	
	3 0		26 2	27	
	4 0		33 2	36	
dl β Hydroxyvaleric	2 5		11 8		12 5
dl β Hydroxyisocaproic	2 0		10 0		10 0
dl β Hydroxycaproic	1 5		21 0	22 5	
dl β Hydroxyoctanoic	1 0		21 4	21 0	
β Ketovaleric	2 0		8 1		8 0
β-Ketoisocaproic	10 0	3 30	13 8		13 2
β Ketooctanoic	7 5	3 84	33 8	38 4	
Isocrotonic	6		62 3	54	
$\Delta^{2,3}$ Pentenoic	5		23 8		25
4 Methyl-$\Delta^{2,3}$ pentenoic	2 5		14		12 5
trans $\Delta^{2,3}$-Hexenoic	2 0		32 8	30 0	
	4 0		62 3	60 0	
cis $\Delta^{2,3}$-Hexenoic	3		41 2	45 0	
Sorbic	5		70	70	

* β Keto acids are not oxidized readily in low concentrations An excess of β keto acid was therefore added and the amount which disappeared during the experiment was determined by analysis

† Theory for *dl* β hydroxy fatty acids calculated on the assumption that both the *d* and *l* forms are oxidized

tested The γ,δ-lactone of triacetic acid, which is the ketonic analogue of sorbic acid, was inactive, whereas sorbic acid was fully active

Both the cis and trans forms of α,β-unsaturated acids are equally active in the two cases in which they have been tested (Table II) In keeping with this lack of specificity towards geometrical isomers is the inability of the oxidase system to distinguish between the *l* and *d* stereochemical forms of β-hydroxy acids Five *dl* acids have been tested, and in each case both isomers were equally oxidized

Substituting a triple bond for a double bond leads to an inactive compound Thus, α,β-hexynoic acid was not oxidized under conditions by which α,β-hexenoic acid was oxidized to completion

Oxidation of Phenyl Fatty Acids—The classical theory of β oxidation is based upon the results of studies with phenyl fatty acids It was therefore of considerable interest to determine whether the kidney enzyme system could oxidize phenyl fatty acids in the manner postulated by Knoop (14, 15) The point was not easy to test because of the intense inhibitory action of phenyl fatty acids on the fatty acid oxidase system Thus, γ-phenylbutyric acid at a concentration of 0 003 M inhibited completely the oxidation of butyric, crotonic, and vinylacetic acids, and with butyric acid was still exerting striking inhibition at a concentration as low as 0 0003 M In order to test oxidizability, γ-phenylbutyric acid had to be diluted to a point at which reliable manometric measurements were no longer possible The same difficulty obtained for phenylpropionic acid and cinnamic acid at one end of the phenyl fatty acid series, and for phenylcapric and phenyllauric acids at the other However, it was possible to demonstrate consistently the oxidation of γ-phenylvaleric and γ-phenyloctanoic acids The rates of oxidation of these two phenyl fatty acids were not sufficiently rapid to permit quantitative studies, and it remains to be determined how far down the carbon chain the oxidation proceeds

Sparking or Priming of Fatty Acid Oxidation by Members of Citric Acid Cycle in Kidney Preparations—Fresh enzymes usually oxidize fatty acids with great rapidity However, when the enzyme is allowed to stand for some hours after preparation, the capacity to oxidize fatty acids is greatly reduced, if not abolished This capacity can be restored by the addition of primers such as α-ketoglutarate or fumarate (Fig 2) Similar effects have already been reported by others (2, 4, 7, 10, 16–19)

The sparking of acetoacetic acid has been studied in some detail At 30 micromoles per cup it is regularly oxidized either very slightly or not at all in the absence of an added sparker, even with the most active enzyme preparations, and just as regularly its oxidation can be sparked by α-ketoglutaric acid Significant effects are obtained with very small amounts of the sparker, and the oxidative rate is sustained for progressively longer periods as the amount of α-ketoglutaric acid is increased (Fig 3) In Fig 3 it will be noted that with 0 5, 1, and 2 micromoles of α-ketoglutaric acid, the reaction having proceeded to virtual completion at 60 minutes, the total oxygen uptake in excess of the appropriate α-ketoglutaric acid blank, and attributable to the oxidation of acetoacetic acid, is in direct proportion to the amount of α-ketoglutaric acid added (0 5 156, 1 301, 2 micromoles 605 c mm of O_2) Extrapolation for the period of equilibration does not seriously affect this proportionality

The following experiment indicates that acetoacetic acid can be completely oxidized to carbon dioxide and water in the presence of α-ketoglutaric acid 10 5 micromoles of acetoacetic acid were added to each of two manometer cups, only one of which was supplemented with 5 micromoles of α-ketoglutaric acid In the cup with the sparker the oxygen uptake, corrected for the blank with acetoacetic acid, corresponded to the complete oxidation of 7 1 micromoles Analysis by the aniline-citrate method (20) showed that 3 6 micromoles of acetoacetic acid remained (theory, 3 4 micromoles) In the cup without an added sparker, the cor-

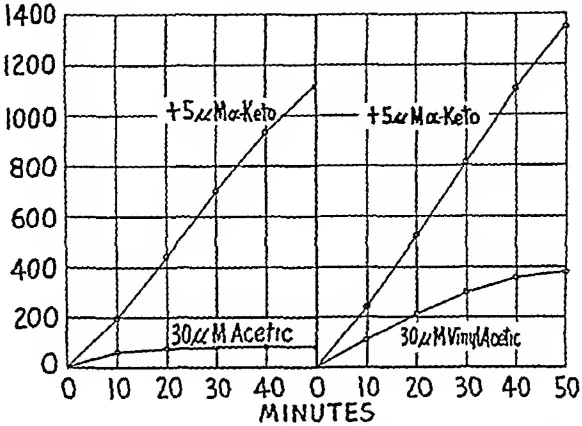

FIG 2 Sparking of acetic acid and vinylacetic acid oxidation by α-ketoglutaric acid The complete system was used with 2 cc of kidney enzyme (R_3K) in the experiment with acetic acid and 1 cc of enzyme in the experiment with vinylacetic acid The enzymes were used $5\frac{1}{4}$ hours and 30 minutes after preparation respectively The oxygen uptake in appropriate controls (without added substrate and with 5 micromoles of α ketoglutaric acid) has been subtracted in all cases, and only the excess oxygen uptake attributable to the oxidation of acetic and vinylacetic acids is shown in the figure The total oxygen uptake in the controls without added substrate was 62 c mm in the experiment with acetic acid, and 53 c mm in that with vinylacetic acid

rected oxygen uptake corresponded to the complete oxidation of only 1 4 micromoles of acetoacetic acid

Although most of the sparking experiments were performed with α-ketoglutaric acid, it was found that all members of the tricarboxylic acid cycle, or substances like glutamate or proline which can give rise to members of the citric acid cycle,[5] could effectively spark the oxidation of acetoacetic acid

The fact that some fatty acids may be readily oxidized at lower concentrations, but oxidized poorly or not at all at higher concentrations, has

[5] Taggart, J V, and Krakauer, R, unpublished experiments

previously been mentioned This inhibition at higher concentrations can frequently be overcome by the addition of small amounts of the sparker A brief description of one experiment will suffice n-Capric acid was rapidly oxidized to completion at 1 25 micromoles and 2 5 micromoles, but was completely inactive at 5 micromoles per cup, on the other hand, 5 micromoles of the acid were oxidized with striking rapidity in the presence of 5 micromoles of α-ketoglutaric acid

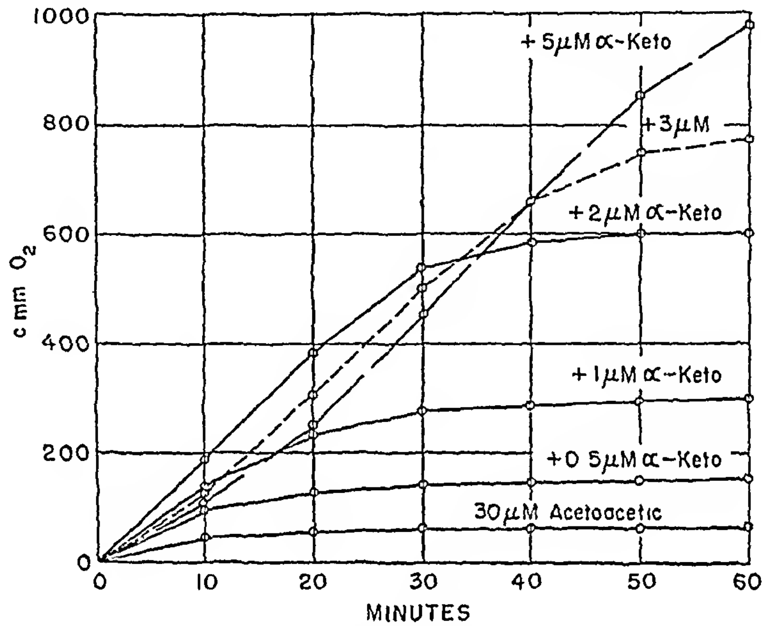

FIG 3 Sparking of acetoacetic acid oxidation by varying amounts of α-ketoglutaric acid The complete system was used with 2 cc of freshly prepared kidney enzyme (R;K) The oxygen uptake in appropriate controls (without added substrate and with 0 5, 1, 2, 3, and 5 micromoles of α-ketoglutaric acid) has been subtracted in all cases, and only the excess oxygen uptake attributable to the oxidation of acetoacetic acid is shown in the figure Oxygen uptake in the control without added substrate was 99 c mm in 60 minutes

It may be pointed out that the conditions of Table I are minimal as far as the amount of the sparker is concerned and that the reproducibility of the rates of oxidation was not too good from one enzyme preparation to another At a higher level of the sparker, reproducibility was excellent All compounds were therefore also tested in the presence of about 5 micromoles of the sparker Whenever a fatty acid is referred to as inactive in this communication, it is implied that the test for activity has also been carried out in the presence of an adequate amount of the sparker

Fatty Acid Oxidation with Ferricyanide—In our previous communication on the cyclophorase system (1), evidence was presented that ferricyanide

could replace oxygen as the oxidizing agent and that ferricyanide oxidation could be followed manometrically by measuring carbon dioxide evolution Ferricyanide has also been found to be effective as an oxidizing agent for fatty acids (Table III) The necessity for the sparking of fatty acid oxidation applies as well to the oxidation by ferricyanide as to the oxidation by molecular oxygen This particular point is discussed by Knox *et al* (11)

Inhibition of Fatty Acid Oxidases—At a concentration of 0 003 M, malonate completely inhibits fatty acid oxidation The same is true for arsenite at the same concentration 0 004 M barium ions, even in the absence of added inorganic phosphate, abolish the oxidation of butyric acid (10 micromoles per cup) Fluoroacetate, which has been extensively investigated

TABLE III

Oxidation of Fatty Acids by Ferricyanide

	Microliters CO_2 per 23 min		Microliters CO_2 per 23 min
Control (no fatty acid)	135	*dl* β Hydroxybutyrate	510
Acetate	513	Acetoacetate	535
Valerate	253	*cis*-Hexenoate	332
β Ketovalerate	433	β-Ketohexanoate	831
Butyrate	634	Sorbate	700
Crotonate	484	Caproate	600
Vinylacetate	524		

Each manometer cup contained 1 5 cc of kidney enzyme at the 3rd residue stage, 0 3 cc of 0 01 M adenosine triphosphate, 0 2 cc of 0 02 M magnesium sulfate, 0 1 cc of 0 5 M sodium bicarbonate, 0 2 cc of 0 5 M sodium ferricyanide, 0 2 cc of 0 01 M α ketoglutarate, and, except for the control, 0 3 cc of 0 1 M fatty acid The gas space was filled with a mixture of 95 per cent nitrogen and 5 per cent carbon dioxide The final volume was made up to 3 0 cc , bath temperature 38°

by Bartlett, Barron, and Kalnitsky (21), is an inhibitor of acetic, butyric, and *n*-caproic acid oxidation, and requires further investigation Propionic acid completely inhibits acetic acid oxidation when the two compounds are present in equimolar concentrations, and markedly inhibits at a concentration one-fourth that of the acetic acid Butyric acid oxidation is abolished by cinnamic acid in equimolar concentration Cinnamic acid, at a concentration one-tenth that of the substrate, inhibited the oxidation of butyric and vinylacetic acids about 25 per cent, while that of crotonic acid was inhibited about 75 per cent γ-Phenylbutyric acid at a concentration of 0 003 M inhibited completely the oxidation of butyric, crotonic, and vinylacetic acids (0 009 M), and striking inhibition of butyric oxidation was observed even at 0 0003 M concentration

Oxidation of Fatty Acids by Liver

Most of our experiments have been carried out with the enzyme system prepared from rabbit kidney, but the salient features of fatty acid oxidation as described above have been found also to apply to the system prepared from rabbit liver Here also the oxidation of fatty acids has to be sparked by some member of the citric acid cycle and this sparked oxidation, under suitable conditions, proceeds to completion, *i e*, to carbon dioxide and water by way of the citric acid cycle (Table IV) The liver fatty acid oxidase system is somewhat less stable than the kidney system, but, providing care is taken to maintain the pH during homogenization, there is not much choice between the two systems

TABLE IV

Oxidation of Fatty Acids in Rabbit Liver System

Details as in Table I, except for the addition of 5 micromoles of α-ketoglutaric acid as the sparker in both the control and the experimental cups The observed oxygen uptake includes the extrapolation correction for the first 5 minutes, during which time the stop-cocks were not closed

Fatty acid	Oxygen uptake in microatoms			Theory for oxidation to isobutyric acid
	Micromoles added	Observed	Theory for complete oxidation	
Isocrotonic	5	38	45	
Butyric	5	53	50	
	5	51 5	50	
Caproic	3	42 5	48	
trans-$\Delta^{\alpha,\beta}$-Hexenoic	3	39	45	
Sorbic	5	63	70	
	7 5	105	105	
Isocaproic	5	32 7		30
Heptylic	2 5	26 2		30
Caprylic	5	77	110	
β-Hydroxycaprylic	5	72	105	

The liver system differs from the corresponding system in kidney in several respects (*a*) It contains enzymes capable of oxidizing propionic or phenylpropionic acid The oxdation of propionic acid proceeds to completion (*b*) The oxidation of isocaproic acid in liver[6] proceeds to completion, *i e* to carbon dioxide and water, and, consistent with that observa-

[6] The propionic, phenylpropionic, and isobutyric oxidases can be demonstrated in liver preparations only at the stage of the 1st or 2nd residues (R_1L, or R_2L) Apparently some factor present in the first discarded supernatant is either essential for these oxidases or is important in their stabilization

tion, it can be shown that isobutyric acid is likewise oxidized to completion By contrast, the kidney system carries the oxidation of isocaproic acid only as far as the stage of isobutyric acid (or propionic acid) (c) Acetic and acetoacetic acids are barely, if at all, oxidized by liver preparations at the stage of the 3rd residue, although these substances are oxidized readily in the kidney system It should be pointed out that the inability to oxidize acetic acid is related to the problem of activating acetic acid and not to the problem of whether activated acetic acid is formed during fatty acid oxidation (d) The liver system has lent itself more readily to the demonstration of the "intermediates," particularly acetoacetic acid, and this property may be interpreted in terms of a disproportion between the amount of fatty acid oxidizing enzymes and the amount of the cyclophorase system (e) The liver fatty acid system at the stage of the 3rd residue is often not complete when fortified with the usual components Some factor which is other than the sparker or adenylic acid, and which is present in an aqueous extract of boiled heart muscle, is needed to restore fully the activity of the liver system

Mechanisms of Fatty Acid Oxidation in Liver and Kidney

The data presented thus far have thrown little light on the mechanism of oxidative degradation of fatty acids, primarily because they deal with conditions under which fatty acid oxidation proceeds to completion, and which, therefore, do not lend themselves to the isolation and demonstration of the "intermediates" Some data are available which bear on the question of mechanism and, while the documentation is still incomplete, certain essential features of mechanism are already recognizable For reasons of simplicity it may be preferable to list separately the events involved in the oxidation respectively of caproic and valeric (or isocaproic) acids

Caproic acid

1 β oxidation to β-ketocaproic acid by way of either α,β unsaturated or β-hydroxy acid
2 Cleavage of β ketocaproic acid to acetic and butyric acids
3 Condensation of acetic and oxalacetic acids to form citric acid
4 β oxidation of butyric to acetoacetic acid
5 Condensation of acetoacetic acid with oxalacetic acid to form citric acid
6 Complete oxidation of citric acid in cyclophorase system

Valeric acid (or isocaproic acid)

1 β oxidation to β ketovaleric acid (or β-ketoisocaproic acid) by way of either α,β unsaturated or β hydroxy acid
2 Cleavage of β-ketovaleric acid (or β-ketoisocaproic acid) to acetic and propionic acids (or isobutyric acid)
3 Condensation of acetic and oxalacetic acids to form citric acid
4 Complete oxidation of citric acid in cyclophorase system

The oxidation of caproic acid differs from that of valeric acid or isocaproic acid in two respects (*a*) it leaves no residue after oxidation and (*b*) it gives rise to acetoacetic acid The reactions of acetoacetic acid will be considered separately from those of other β-keto acids, although the mechanisms may well be identical

TABLE V

Acetoacetic Acid Formation during Fatty Acid Oxidation in Liver

Each of the fatty acids was tested in the presence of 1 5 cc of liver enzyme at the R_1 residue stage, 0 6 cc of 1 per cent heart extract, and 0 3 cc of 0 01 M α-ketoglutarate Final volume 3 cc, oxygen in the gas space 38° The value for acetoacetic acid found at the end of the experiment in the blank was usually about 1 micromole or less

Experiment No	Fatty acid	Micromoles added	Microatoms oxygen absorbed (corrected for blank)	Micromoles acetoacetic acid formed (corrected for blank)
1	Caproic	40	163	14 8
	β-Hydroxycaproic	40	124	13 1
	cis-$\Delta^{1,2}$-Hexenoic	40	30	1 0
	trans-$\Delta^{1,2}$-Hexenoic	40	119	15 1
	Sorbic	40	84	12 5
2	Butyric	50	40	5 4
	Crotonic	50	21	6 6
	Isocrotonic	50	34	6 6
	Vinylacetic	50	32	7 2
	β-Hydroxybutyric	50	22	13 6
3	Caproic	40	154	15 0
	Octanoic	20	132	12 8
	Valeric	60	79	2 1
	Heptylic	30	114	3 8
	Isocaproic	60	93	1 4
4	Caproic	40	163	14 8
	Valeric	60	99	0 4
	α,β-Pentenoic	60	75	0 3
	Isocaproic	60	80	1 1
	4 Methyl-$\Delta^{1,2}$-pentenoic	60	105	0 4

Evidence for β Oxidation—The results of Table II show conclusively that all the postulated "intermediates" formed during β oxidation of fatty acids in the kidney system are oxidized to the same products and at approximately the same speed as the parent fatty acids By contrast, no α- or γ-substituted fatty acids were found to undergo oxidation The liver system provides further confirmation of β oxidation Under the conditions obtaining in Experiments 1 to 4 summarized in Table V, even numbered fatty acids from C_4 to C_8 are found to give rise to considerable amounts of acetoacetic acid Significantly, the postulated "intermediates" of β oxida-

tion of these fatty acids give rise to approximately the same amount of acetoacetic acid as the parent fatty acids It is to be noted that the odd numbered fatty acids or isocaproic acid, although oxidized readily under the same experimental conditions, give rise to little if any acetoacetic acid[7] Thus acetoacetic acid in this series of experiments must be presumed to arise exclusively from butyric acid as an intermediate and the conclusion is permissible that caproic and octanoic acids as well as their corresponding products of β oxidation are oxidized to acetoacetic acid via butyric acid as an "intermediate"

The above evidence is only indirect in that it does not establish the formation of β-hydroxy, α,β-unsaturated acids, or β-keto acids as "intermediates" of β oxidation, but merely shows that these derivatives behave as true "intermediates" Acetoacetic acid is the one "intermediate" of β oxidation which has been established by direct means

Table VI

Oxidation of β-Hydroxy Acids by Liver System

Acid	Microatoms oxygen taken up (blank subtracted)	β Keto acid formed	Micromoles keto acid formed (blank subtracted)
dl-β Hydroxybutyrate	38 2	Acetoacetic	21 1
dl β Hydroxyvalerate	22 8	β-Ketovaleric	15 1
dl β-Hydroxycaproate	24 0	Acetoacetic	3 9
dl-β Hydroxyoctanoate	19 7	"	3 4
dl β-Hydroxyisocaproate	0 3		0 6

Each manometer cup contained 1 5 cc of rabbit liver enzyme at the 3rd residue stage, 0 2 cc of 0 01 M adenylic acid, 0 1 cc of 0 125 M phosphate buffer of pH 7 2, 0 1 cc of 0 02 M magnesium sulfate, and 1 cc of 0 1 M *dl* β hydroxy acid Final volume 3 0 cc, alkali in the center well, 38°

There is present in both the liver and kidney fatty acid oxidizing systems an enzyme which catalyzes the oxidation of β-hydroxybutyric acid and some of its higher homologues to the corresponding keto acids This oxidase is unique among the fatty acid oxidizing enzymes in that its substrate does not have to be activated To what extent this enzyme participates in fatty acid oxidation is uncertain, but at any rate it provides the most direct demonstration of one phase of β oxidation The data of Table VI show

[7] The fact that odd numbered fatty acids do not give rise to acetoacetic acid makes it necessary to distinguish this rabbit liver system from the rat liver system of Lehninger (9) in which acetoacetic acid rises equally well from odd as from even numbered fatty acids Acetoacetic acid accumulates in the crude rabbit liver system apparently because of the presence of some substance or substances which inhibit the subsequent oxidation of acetoacetic acid In more purified rabbit liver systems, acetoacetic acid fails to accumulate to any significant amount

the action of the β-hydroxy acid oxidase of the liver system on several β-hydroxy acids It will be noted that no sparker has to be provided for these oxidations to proceed Only in the case of β-hydroxyvalerate does the ratio, microatoms of oxygen absorbed to micromoles of keto acid formed, approach the theoretical value of 1 In some experiments the theoretical values for β-hydroxyvalerate and butyrate have indeed been attained, but under the same conditions the yield of keto acid resulting from the oxidation of β-hydroxycaproate and β-hydroxyoctanoate has been almost negligible This would suggest that in the latter two cases oxidation proceeds beyond the keto acid stage As a matter of fact the corresponding keto acids are not formed, but instead acetoacetic acid[8] accumulates No success has attended efforts to stop the oxidation of β-hydroxycaproate and β-hydroxyoctanoate at the stage of the corresponding β-keto acids The only β-keto acids which appear to be stable under the conditions of the experiment are acetoacetic acid and β-ketovaleric acid Coincident with the oxidation of hydroxy acids there is esterification of inorganic phosphate and it may well be this circumstance which explains why the β-keto acids formed in the oxidation undergo further transformation, even though added β-ketohexanoic and β-ketooctanoic acids are immune to oxidation under the same conditions The β-hydroxy acid oxidase appears to be a self-sparking system like the oxidase which oxidizes pyruvic acid to acetoacetic acid

The oxidation of the β-hydroxy acid to the keto acid is a reversible one As shown in Table VII, the reduction of various β-keto acids can be linked with the oxidation of α-ketoglutaric acid to succinic acid and carbon dioxide The oxidation-reduction reaction can be followed manometrically by measuring carbon dioxide formation The reversible nature of the hydroxy acid oxidase system can also be demonstrated with the oxidation-reduction indicator, benzyl viologen In the presence of β-hydroxybutyrate and under anaerobic conditions, the indicator is reduced to the blue semiquinoid form, whereas, when acetoacetate is introduced into the enzyme mixture from a side arm, the blue color is rapidly discharged

Cleavage of β-Keto Acids to Acetic Acid and Residue Fatty Acid—This phase of fatty acid oxidation is now readily demonstrable Valeric acid

[8] The rate with which β-keto acids are catalytically decomposed by aniline varies inversely with the length of the carbon chain By carrying out the rate studies under standard conditions it becomes possible to determine whether acetoacetic acid or a higher β-keto acid is present in the medium The method is applicable or reliable when only one β-keto acid is present Rate studies applied to the identification of the β-keto acids which accumulated during the oxidation of β-hydroxycaproic and octanoic acids showed unequivocally that the curves were superimposable with that of acetoacetic acid and deviated very widely from those of the higher β-keto acids However, the formation of β-ketovalerate from β-hydroxyvalerate was confirmed by the analysis of the curve for catalytic decomposition

(or β-ketovaleric acid) on oxidation gives rise to propionic acid, which has been identified and characterized by the counter-current distribution method Similarly, isocaproic acid (or β-ketoisocaproic acid) gives rise to isobutyric acid These two end-products could arise only as the result of a cleavage reaction leading to the formation of acetic acid In that connection the inactivity of isovaleric acid is significant and isovaleric acid can be excluded as an "intermediate" in the oxidation of isocaproic to isobutyric acid

The cleavage of β-keto acids into acetic acid is best shown by experiments in which acetic acid is trapped, so to speak, by condensation with oxalacetic acid The extra formation of citric acid from oxalacetic acid in the presence

TABLE VII

Reduction of β-Keto Acids by α-Ketoglutaric Acid

Addition	Microliters CO_2 in 30 min
α-Ketoglutarate	0
" + acetoacetate	111
" + β-ketovalerate	93
" + β-ketohexanoate	85
" + β-ketoisocaproate	44
" + β-ketooctanoate	3
Acetoacetate (no α ketoglutarate)	0

Each manometer cup contained 1 5 cc of kidney enzyme at the R_3 residue stage, 0 3 cc of 0 01 M adenylic acid, 0 2 cc of 0 02 M magnesium phosphate, 0 2 cc of 0 125 M phosphate buffer of pH 7 3, 0 1 cc of 0 5 M sodium bicarbonate, 0 3 cc of 0 1 M α-ketoglutarate, and 0 5 cc of 0 1 M *dl* β-hydroxy acid 95 per cent nitrogen, 5 per cent carbon dioxide gas mixture in the air space

of β-keto acids (Tables VIII and IX) generally provides the most direct evidence that acetic acid arises during the breakdown of β-keto acids These results are in agreement with the experiments reported by Breusch (24), Wieland and Rosenthal (25), and Hunter and Leloir (23) This Breusch effect (17) might be interpreted in terms of the β-keto acids acting as hydrogen acceptors However, this interpretation can be ruled out, since (*a*) the experiments are carried out in oxygen, (*b*) oxygen is needed for the reaction, and (*c*), as shown in Table IX, β-hydroxybutyric acid can replace acetoacetic acid quantitatively in this reaction

Condensation of Acetic Acid and Oxalacetic Acid to Form Citric Acid—It can readily be demonstrated that acetic acid is not directly oxidized in the kidney enzyme system Every conceivable stable "intermediate" such as glycolic acid, oxalic acid, glyoxylic acid, and glycolaldehyde has been tested and found to be inactive The alternative to direct oxidation is, of

course, by way of condensation with oxalacetic acid and then by entry into the reactions of the cyclophorase system

The use of isotopes has been essential to decide whether acetic acid can condense with oxalacetic acid to form citric acid The isotope experiments[9] will be presented in a later communication

β Oxidation of Butyric Acid to Acetoacetic Acid—The data of Table IV establish the formation of acetoacetic acid from butyric acid or from fatty acids which give rise to butyric acid

TABLE VIII

Citric Acid Formation from Condensation of Oxalacetic and Acetoacetic Acids

The system contained 1 cc of enzyme at the R_3K residue stage, 0 05 cc of M barium chloride, and 0 1 cc of 0 02 M magnesium sulfate Final volume, 3 0 cc Experiment carried out in air at 38° for 1 hour Citric acid was estimated by the method of Pucher *et al* (22) as modified by Hunter and Leloir (23) Every estimation of an experimental solution was carried out with a parallel estimation of the boiled enzyme control for that particular solution The above results are already corrected for the "citric acid" found in the control solutions with boiled enzyme The control values never exceeded 1 micromole of citric acid

The results are expressed in micromoles

System with	Experiment 1 70 micromoles oxalacetic, 30 micro moles acetoacetic	Experiment 2 60 micromoles oxalacetic, 30 micro moles acetoacetic	Experiment 3 40 micromoles oxalacetic, 25 micro moles acetoacetic	Experiment 4 40 micro moles oxalacetic, 25 micro-moles acetoacetic	Experiment 5 70 micro moles oxalacetic, 30 micro moles acetoacetic
No additions	0 4	0 2	0 2	0 3	0 3
Oxalacetic acid	1 2	5 3	3 8	3 5	2 6
" and acetoacetic acids	7 9	10 3	11 2	9 2	11 6
Acetoacetic acid	0 4			0 4	0 3

Condensation of Acetoacetic Acid and Oxalacetic Acid to Form Citric Acid—Table VIII has already summarized the evidence on this point The interaction of acetoacetic and oxalacetic acids may be considered to be the result of two separate reactions (1) cleavage of activated acetoacetic into 2 molecules of acetic acid, and (2) condensation of activated acetic acid with oxalacetic acid Acetoacetic acid does not give rise to acetic acid unless activated

The yield of citric acid from a mixture of oxalacetic and acetoacetic acid is usually at least double that from oxalacetic acid alone, whereas the yield from a mixture of oxalacetic and acetic acid is rarely greater than that from oxalacetic acid alone

Complete Oxidation of Citric Acid in Cyclophorase System—In our previous communication the complete oxidation of citric acid or of any of its

[9] Knox, W E, and Weinhouse, S, unpublished experiments

related forms to carbon dioxide and water has already been established for the kidney cyclophorase system The conditions which favor the preparation of an active cyclophorase system are identical with those which favor the preparation of active fatty acid systems Both the cyclophorase system and the fatty acid oxidase system require magnesium ions, adenylic acid (or adenosine triphosphate), and phosphate ions for maximal activity The main distinction is that the cyclophorase system is complete when provided with any member of the citric acid cycle and the above components

TABLE IX

Citric Acid Formation from Oxalacetic Acid and β-Keto Acids

Additions	Micromoles citric acid formed	Additions	Micromoles citric acid formed
None	0 2	None	0 2
Oxalacetic	6 3	Oxalacetic	4 9
" + β ketovaleric	10 3	" + acetoacetic	11 3
β-Ketovaleric	0 4	" + β hydroxybutyric	10 4
Oxalacetic + β hydroxybutyric	10 0	Oxalacetic + β ketovaleric	9 1
β-Hydroxybutyric	0 2	" + β-ketohexanoic	9 2
		Oxalacetic + β-ketooctanoic	5 8

Each manometer cup contained 1 5 cc of kidney enzyme at the 3rd residue stage, 0 3 cc of 0 01 M adenylic acid, 0 2 cc of magnesium sulfate, 0 3 cc of 0 125 M phosphate buffer of pH 7 2, and 0 1 cc of M barium chloride Where indicated, 0 6 cc of 0 1 M oxalacetate and 0 6 cc of 0 1 M β keto acid or hydroxy acid were used The experiments were carried out in oxygen gas at 38°

The fatty acid system does not start to function under the same conditions unless the cyclophorase system is also functioning initially

Activation of Fatty Acids and "Intermediates"—The discussion of the mechanism of fatty acid oxidation in the liver and kidney systems would be incomplete without reference to the problem and nature of the so called sparking or activation phenomenon Another communication will be reserved for the systematic consideration of oxidative phosphorylation and the sparking phenomenon For present purposes it will be sufficient to state that fatty acids and their products of oxidation are not oxidized as such but only after combination with what appear to be pyrophosphate-containing coenzymes as yet unspecified

EXPERIMENTAL

Enzyme suspensions were prepared from rabbit kidneys as previously described, with the addition of alkali during homogenization with 0 9 per

cent potassium chloride (1) Unless otherwise specified, a suspension in dilute salt solution of the 3rd residue, designated R_3K (the residue from centrifugation of the homogenate twice resuspended and washed with potassium chloride), was used in all the experiments, which were performed in Warburg manometers at 38° The enzyme (1, 1 5, or 2 cc per cup) was routinely supplemented by the following additions 0 3 cc of 0 01 M adenosine triphosphate, 0 2 cc of 0 02 M magnesium sulfate, and 0 5 cc of 0 04 M phosphate buffer of pH 7 3, and in most instances by the further addition of 0 3 cc of a 1 per cent aqueous extract of acetone powder of pig heart, acidified to about pH 6 with HCl (for preparation, see Knox *et al* (11)) A gas phase of 100 per cent O_2 was used, as this was found to increase oxidative rates markedly above those observed in air Controls without added substrate were included in all experiments All the substrates were added in solution as neutral sodium salts

Butyric, valeric, isovaleric, capric, lauric, myristic, phenylpropionic, and cinnamic acids were obtained from the Eastman Kodak Company Pure samples of *n*-caproic, *n*-caprylic, stearic, oleic, and azelaic acids were generously provided by Dr David Rittenberg The Armour Laboratories kindly provided *n*-heptylic, *n*-undecylic, and *n*-tridecylic acids *trans*-$\Delta^{\alpha,\beta}$-Hexenoic and styrylacrylic acids were the gifts of the Carbide and Carbon Chemicals Corporation The Fairchan Laboratories prepared for us highly purified and authentic samples of phenyloctanoic, phenylcapric, phenyllauric, acrylic, 3,4-epoxybutyric, *cis*-$\Delta^{\alpha\ \beta}$-hexenoic, isocrotonic, $\Delta^{2\ 3}$-pentenoic, $\Delta^{4,5}$-pentenoic, dimethylacrylic, and 4-methylpentenoic acids, also the ethyl esters of β-hydroxyvaleric, β-hydroxyhexanoic, β-hydroxyisocaproic, β-hydroxyoctanoic, β-hydroxylauric, β-ketoisocaproic, and β-keto-octanoic acids Dr Herbert E Carter generously provided us with samples of various alkyl- and phenyl-substituted fatty acids, with the "intermediate" for the preparation of phenylvaleric acid, and with a pure sample of *dl*-threo-2,3-dihydroxybutyric acid

Acetoacetic acid was prepared by hydrolysis of the ethyl ester with the theoretical quantity of alkali, followed by the removal of ethyl alcohol by vacuum distillation The same procedure was applied to the preparation of the other β-keto and β-hydroxy acids from their respective ethyl esters *dl*-β-Hydroxybutyric and *dl*-β-hydroxycaproic acids were prepared from the corresponding keto acids by reduction with sodium amalgam in alkaline solution γ-Hydroxybutyric acid (γ-butyrolactone), succinic semialdehyde, and vinylacetic acids were prepared by the methods of Sircar (26), Dakin (27), and Falaise and Frognier (28) respectively Vinylacetic acid was purified according to Rietz (29) β-Benzoylpropionic and γ-phenylbutyric acids were synthesized by the methods given in "Organic syntheses" (30) β-Ketovaleric and β-ketocaproic ethyl esters were prepared according to the method of Fischer *et al* (31)

All the keto acids tested were estimated manometrically as carbon dioxide by the aniline-citrate manometric method (20) Succinic semialdehyde was estimated by the bisulfite method of Clift and Cook (32)

SUMMARY

The complete oxidation of fatty acids and their derivatives to carbon dioxide and water has been studied in kidney and liver cyclophorase preparations

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STUDIES ON THE CYCLOPHORASE SYSTEM

III OBLIGATORY SPARKING OF FATTY ACID OXIDATION

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The fatty acid oxidizing system, prepared from washed rabbit kidney as described in the previous paper (1), often did not oxidize fatty acids unless supplemented by a small amount of one of the cyclophorase substrates A similar effect could be obtained by adding an extract of boiled heart muscle This addition was regularly found to be necessary for fatty acid oxidation by an enzyme at the 3rd residue (R_3) stage which had been prepared with precautions to insure thorough washing at each centrifugation The effects of fumarate or of heart extract addition on such an enzyme are comparable (Fig 1) Virtually no oxidation of butyrate occurs in the absence of any addition, whereas a maximal rate of butyrate oxidation is achieved by the addition of an amount of either substrate or heart extract which in the absence of butyrate is responsible for an insignificant oxygen uptake Suboptimal additions of either produce proportionally lower rates of butyrate oxidation Other substrates directly oxidizable by the cyclophorase system, when tested in comparable concentrations, were found to produce the same effect as fumarate and heart extract

All the known coenzymes were assayed in this system for activity in initiating oxidation of butyrate[1] The slight activity, found in several of the preparations which were not available in pure form, disappeared upon further purification Only glutathione was active, producing an effect equal to that of fumarate or glutamate on a molar basis (Table I) The glutamic acid moiety of glutathione is oxidized by the cyclophorase system Analysis of a sample of heart extract[2] showed 0 05 micromole of glutathione

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[1] The following compounds were tested coenzyme I, coenzyme II, flavin adenine dinucleotide, riboflavin monophosphate, cytochrome *c*, adenosine triphosphate (ATP), adenosine 3-phosphate, thiamine pyrophosphate, pyridoxal phosphate, glutathione, coenzyme of acetylation (from Dr David Nachmansohn), vitamin B_c conjugate, and pteroylglutamic acid

[2] The heart extract was prepared as follows 1 kilo of fresh pig heart was trimmed, minced in the cold, and extracted after 10 minutes with 2 volumes of water at 95–100°

per mg of dry weight of extract, determined iodometrically (2), and 0 16 micromole per mg of succinate plus glutamate, determined enzymically

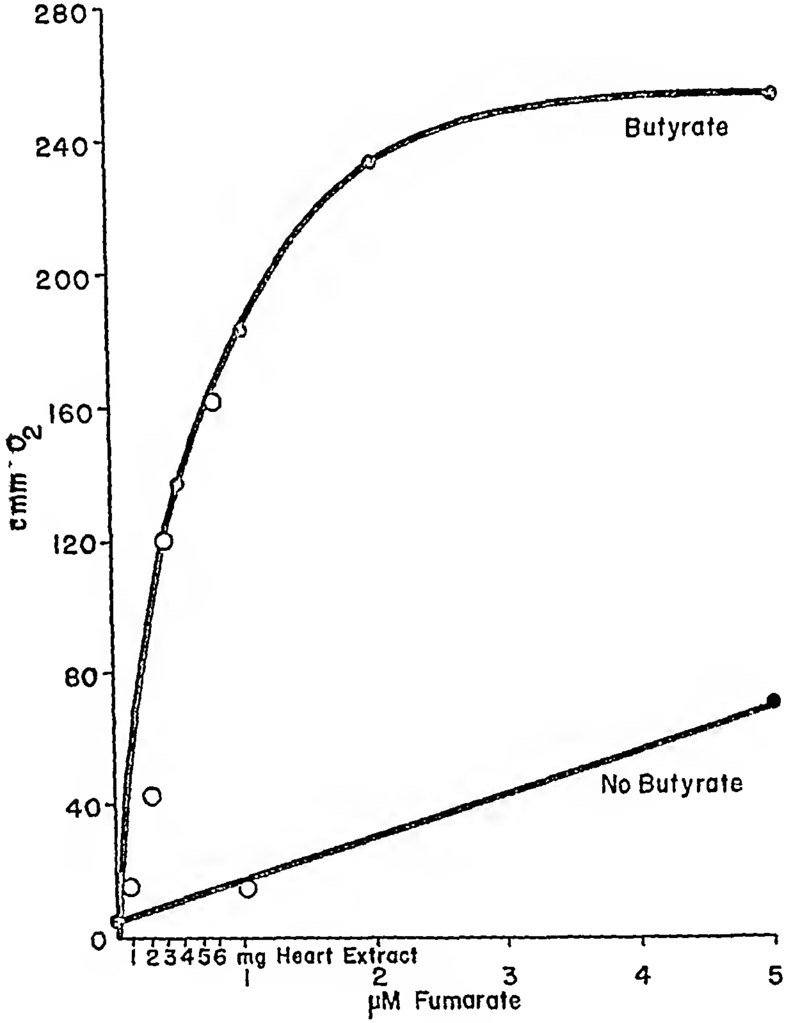

FIG 1 Sparking of butyrate oxidation by fumarate and by heart extract Each cup contained 1 cc of kidney enzyme at the 3rd residue stage (R_3) 3½ hours old, 0 3 cc of 0 01 M ATP, 0 2 cc of 0 02 M $MgSO_4$, 0 5 cc of 0 04 M phosphate buffer, pH 7 25, total volume 3 0 cc , O_2 in the gas phase, NaOH in the center well, equilibrated at 38° for 7 minutes before closing the taps Fumarate (o) or heart extract (O) in the amounts shown, and 0 3 cc of 0 1 M butyrate where indicated, were added to the cups beforehand The oxygen uptake was recorded during the first 10 minutes after equilibration

after chloramine-T oxidation (3) This heart extract, which thus contained at least 0 21 micromole per mg of oxidizable substrates, had the same effect

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The aqueous extract after chilling was precipitated with 4 volumes of acetone and the precipitate dried with acetone and ether

per mg on butyrate oxidation as 0 25 micromole of substrate Most, if not all, of the effect of the heart extract can therefore be attributed to the

TABLE I

Sparking of Butyrate Oxidation by Fumarate, Glutamate, and Glutathione

Same conditions as in the legend of Fig 1 One enzyme preparation was used in the comparison of fumarate with glutamate and another preparation for the comparison of fumarate with glutathione

Addition	Amount	O_2 per 5 min	Addition	Amount	O_2 per 10 min
	micro mole	*c mm*		*micro mole*	*c mm*
		8			12
Fumarate	0 4	29	Fumarate	0 2	30
Glutamate	0 4	18	Glutathione	0 2	19
Fumarate	0 8	93	Fumarate	0 4	84
Glutamate	0 8	90	Glutathione	0 4	82
Fumarate without butyrate	0 8	7	Fumarate	0 8	107
Glutamate without butyrate	0 8	8	Glutathione	0 8	126
			Fumarate without butyrate	0 8	10
			Glutathione without butyrate	0 8	10

TABLE II

Comparison of Amount of Fumarate Necessary to Initiate Oxidation of Some Fatty Acids

The oxygen uptake, without the subtraction of the blanks, is given for the fatty acids alone and with added fumarate, and was determined under the conditions given in the legend of Fig 1

Fatty acid added	C.mm O_2 per 15 min		
	Fatty acid alone	+ 0.5 micro mole fumarate	+ 3 0 micromole fumarate
Butyrate, 30 micromoles	25	218	347
Octanoate, 5 "	12	158	
" 20 "	13	24	174
Acetate, 30 micromoles	20	28	151
Acetoacetate, 30 micromoles	17	28	83
No fatty acid	15	18	60

presence of traces of these three substrates directly oxidizable by the cyclophorase system

Amount of Cyclophorase Substrate Required to Initiate Oxidation of Different Fatty Acids—In the initial phase of these investigations, two types of oxidative reactions involving fatty acids were distinguished, depending upon whether or not additional cyclophorase substrate was required How-

ever, even in the case in which no substrate had to be added, the test system contained heart extract, which served as a minimal source of substrate The difference originally observed between various fatty acids is one of degree All require some substrate to initiate their oxidation, but some fatty acids require more than others (Table II) These necessary amounts of substrate may be added directly or in the form of heart extract, or may already be present in the trace amounts required for certain reactions in less well washed enzyme preparations By controlling these factors, it has been possible to show that oxidation of all the fatty acids and fatty acid derivatives which we have studied must be sparked by the simultaneous oxidation of a small amount of a cyclophorase substrate There is no evidence that an unsparked oxidation of fatty acids can occur The amount required is characteristic of the conditions used and of the fatty acid oxidized, and bears no relation to the total amount of fatty acid that can be oxidized once the reaction begins

In consequence of this obligatory nature of the sparking reaction, the fact that butyrate and octanoate can frequently be oxidized to completion under conditions in which added acetoacetate is not oxidizable is no bar to accepting acetoacetate as an intermediate All three compounds require sparking, though in different amounts, and all three compounds, when changed by the sparking reaction, may share a common pathway

Nature of Sparking Reaction

All attempts to initiate the oxidation of fatty acids in some way not involving simultaneous oxidation of a cyclophorase substrate have been unsuccessful There is no evidence, for example, that an initial anaerobic condensation occurs between the fatty acid and pyruvate or oxalacetate Although added $NaHCO_3$ will often increase the blank oxygen uptake of enzyme at the 3rd residue stage (R_3) and initiate fatty acid oxidation, this is due to the presence in the enzyme of a small amount of pyruvate, which with the added CO_2 can be oxidized through its conversion to oxalacetate (4) Further washing to remove these traces of pyruvate prevents this artifact of "sparking with CO_2" (Table III) As would be expected, such a pyruvate-free enzyme then requires a larger amount of substrate to initiate fatty acid oxidation Finally, none of the many fatty acid derivatives tested can be oxidized directly without sparking These cannot therefore be identical with the intermediates produced by the sparking reaction

In the previous communication (1) the use of ferricyanide as an oxidizing agent for fatty acid oxidation was described It was of considerable interest to determine whether the sparking phenomenon would apply when ferricyanide replaced oxygen as oxidizing agent The data of Table IV establish that the sparking phenomenon is concerned with the primary

dehydrogenation of the cyclophorase substrate, and not with the subsequent hydrogen transport through the systems reacting with oxygen

The initiation of fatty acid oxidation is obligatorily associated with the simultaneous oxidation of a small amount of a cyclophorase substrate The oxidations of the several substrates which can spark fatty acid oxidation all produce esterification of phosphate Of particular interest is the effect of

TABLE III

Effect of Added CO_2 on Butyrate Oxidation by Kidney Enzyme Preparations at 3rd and 5th Residue Stages

0 2 cc of 0 1 M $NaHCO_3$ was added as indicated The conditions are otherwise as in the legend for Fig 1 The values are c mm of O_2 per 15 minutes

Enzyme	CO_2 only	Butyrate only	CO_2 + butyrate	0 6 micromole fumarate only	Butyrate + 0 6 micromole fumarate	Butyrate + 3 0 micromoles fumarate
R_3K	25	19	165	12	114	
R_5K	17	17	23	13	12	194

TABLE IV

Sparking of Fatty Acid Oxidation in System containing Ferricyanide As Oxidizing Agent

Each cup contained 1 5 cc of kidney enzyme at the 3rd residue stage, 0 2 cc of 0 02 M magnesium sulfate, 0 3 cc of 0 01 M adenosine triphosphate, 0 1 cc of 0 5 M sodium bicarbonate, and 0 2 cc of 0 5 M sodium ferricyanide Total volume, 3 0 cc Gas space filled with 95 per cent nitrogen 5 per cent carbon dioxide

Additions	C mm CO_2 1st 10 min	C mm CO_2 2nd 10 min
None	6	4
α Ketoglutarate (2 micromoles)	59	45
Butyrate (10 micromoles)	40	21
" (10 ") + α ketoglutarate (2 micromoles)	272	241
cis-Hexenoate (10 micromoles)	44	24
" (10 ") + α ketoglutarate (2 micromoles)	226	218

dinitrophenol and gramicidin on these oxidations Dr J V Taggart and his colleagues have shown that these two reagents prevent esterification of inorganic phosphate without inhibiting the oxidation of the cyclophorase substrates They also completely prevent fatty acid oxidation even when a substrate of the cyclophorase system is undergoing rapid oxidation However, the energetic coupling of the initial fatty acid reaction with the oxidation of the cyclophorase substrates suggested by these observations cannot be attributed simply to the generation of ATP (5) ATP, in an amount greater than can be destroyed during the experiment, does not

replace an oxidizable substrate as the sparker Similarly, the acyl phosphates of the fatty acids, which have been suggested as the active form for oxidation (5, 6), cannot be oxidized in this system without sparking During the sparked oxidation of a fatty acid, no acyl phosphate accumulates that can be determined by Lipmann and Tuttle's hydroxylamine reaction (7)

The initial reaction of a fatty acid has therefore not yet been dissociated from the simultaneous oxidation of a directly oxidizable substrate Another function of this added substrate, in addition to sparking, must also be that of condensing with activated acetate and acetoacetate formed during fatty acid oxidation By this condensation fatty acids may be oxidized to completion through the citric acid cycle, as will be shown by isotopic experiments to be published later A reaction analogous to this, the condensation of acetoacetate with oxalacetate to form citric acid, has already been demonstrated in this system (1)

SUMMARY

Oxidation of fatty acids and their derivatives by the enzymes from rabbit kidney can occur only if a small amount of cyclophorase substrate is first oxidized to initiate the reaction This sparking effect by compounds of the citric acid cycle consists of a primary activation of the fatty acid which is not duplicated by ATP or acyl phosphates, and of a later condensation between a cyclophorase substrate and the "acetate" leading to complete oxidation through the cycle

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STUDIES ON THE CYCLOPHORASE SYSTEM

IV DIRECT DEMONSTRATION OF β OXIDATION

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In a previous communication of this series (1), manometric evidence was presented which showed that normal fatty acids with an odd number of carbon atoms and fatty acids of the *iso* series with an even number of carbon atoms were not completely oxidized to carbon dioxide and water The quantitative data were consistent with the formation of propionic acid in the former case and of isobutyric acid in the latter case as non-oxidizable residues This interpretation became even more plausible in view of the observation that both propionic acid and isobutyric acid appeared not to be oxidized by the kidney enzyme system The possibility of identifying these hypothetical residues arising from the oxidation of their parent fatty acids provided the opportunity for a direct demonstration of β oxidation According to Knoop's classical theory (2) fatty acids are degraded by successive scissions of a 2-carbon unit from the main chain Accordingly, valeric acid should give rise to propionic acid and isocaproic acid should give rise to isobutyric acid If, in addition, it could be shown that butyric acid is not an intermediate in the oxidation of valeric acid nor isovaleric acid in the oxidation of isocaproic acid, the evidence for β oxidation would be complete This communication deals with the demonstration of these points plus the demonstration of propionic acid as an unexpected oxidation product of isobutyric acid

The resolution and characterization of the fatty acids studied were effected by the method of counter-current distribution as developed by Craig and his coworkers (3-6) The underlying principle of this technique is that compounds with different coefficients of distribution between two immiscible solvents will each exhibit a characteristic behavior when subjected to what is in essence a graded series of extractions in a system of separatory funnels Not only does this behavior lend itself to the separation of the components of a mixture, but it also permits calculation of the coefficient of distribution for each component Identification may then be made by comparison of these derived coefficients with those of known

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compounds, and, conversely, knowing the distribution coefficient of a pure substance, one can accurately predict its behavior in the Craig apparatus for comparison with the observed behavior of an unknown substance

Results

Oxidation of Valeric Acid—The oxidation of valeric acid with the kidney enzyme system was carried out on a large scale with α-ketoglutaric acid as a cooxidant (*cf* "Experimental" for details) After the reaction was over, as judged from a small pilot run, the enzyme suspension was deproteinized and the volatile fatty acids separated by distillation from acid solution The spectrum for the counter-current distribution of the fatty acids is shown in Fig 1.

The presence of propionic acid is indicated by the curve with its peak in Tube 8, for one can see that its observed values resemble closely those calculated for a pure substance with a distribution coefficient of 0 53, which figure a previous distribution of pure propionic acid had shown to be the distribution coefficient for this substance in this particular solvent system

The remainder of the valeric acid added originally is represented by the curve with its peak in Tube 22 The observed values were found to fit best a theoretical curve calculated for a substance with a K of 11 3, while the K of a known pure sample of valeric acid had previously been shown to be 9 7 in this system [1]

A blank run identical with that of the experimental run, save that it was precipitated immediately after the addition of the enzyme, showed the presence of large amounts of valeric acid but no propionic acid

The propionic acid which has been shown to accumulate from the oxidation of valeric acid must have resulted from the splitting off from the parent compound of a 2-carbon unit, for repeated studies on the oxidation of butyric acid by this enzyme system with manometric techniques have shown that this compound is oxidized to completion (1) and thus could not serve as an intermediate in the oxidation of a substance which gives rise to a non-oxidizable residue

Accumulation of Acetic Acid—A brief digression is necessary here to explain the peak which is seen at the far left of Fig 1 This peak was found to be present also in all subsequent experiments and usually corresponded to

[1] At either end of the distribution spectrum it must be noted that the distribution coefficient represents the quotient of a relatively large number divided by a relatively small number, or *vice versa*, and experimental errors are magnified accordingly In the studies described here, reproducibility of values for K was poor at the ends of the spectrum It will be seen, however, that those substances which require the most precise identification all lie sufficiently near the center of the spectrum for consistently accurate results

one which would be given by a substance having a distribution coefficient of about 0 060 to 0 10 Since acetic acid could be shown to have a K of about 0 065 in this solvent pair,[1] this was taken as presumptive evidence that the peak did represent acetic acid In a later run in which the peak

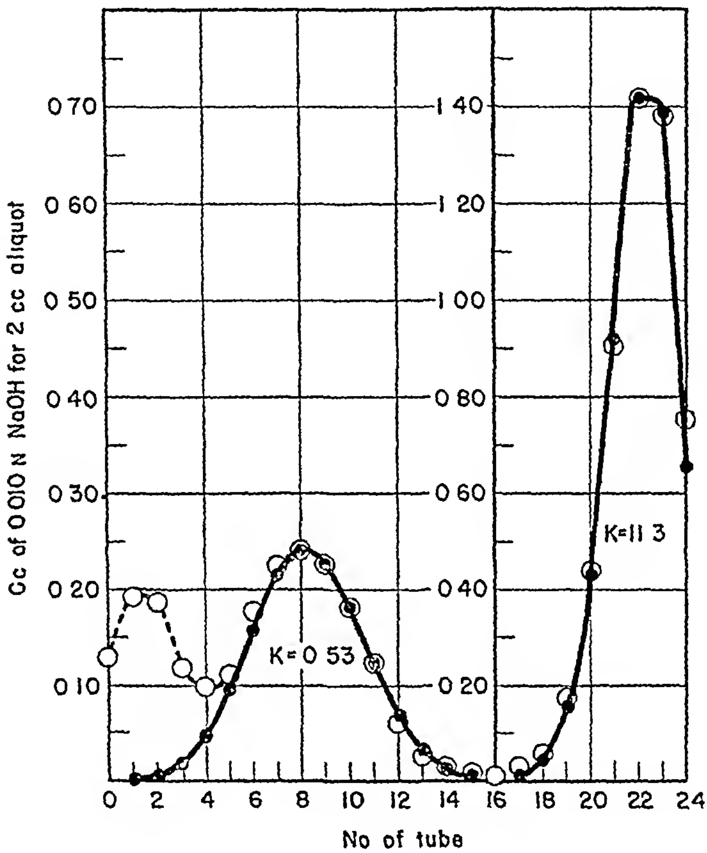


FIG 1 Oxidation of valeric acid Solvent system, isopropyl ether and 2 2 M phosphate buffer of pH 5 19 The titration values are for 2 cc aliquot of the initial extraction 400 micromoles of valerate were present at the start of the reaction, 214 were present finally Propionic peak represents about 99 micromoles O, observed values, ●, calculated values for K of 0 53 and 11 3, as indicated

appeared quite free of any contamination by propionic acid, the contents of Tubes 0 to 4 were pooled and the fatty acid separated by distillation In 0 1 M solution the sodium salt of the fatty acid reacted positively in the lanthanum nitrate test, thus yielding further evidence that the compound responsible for the peak actually was acetic acid The presence of this

fatty acid was shown by counter-current techniques not to be due to impurities already present in the reagents used in these experiments It

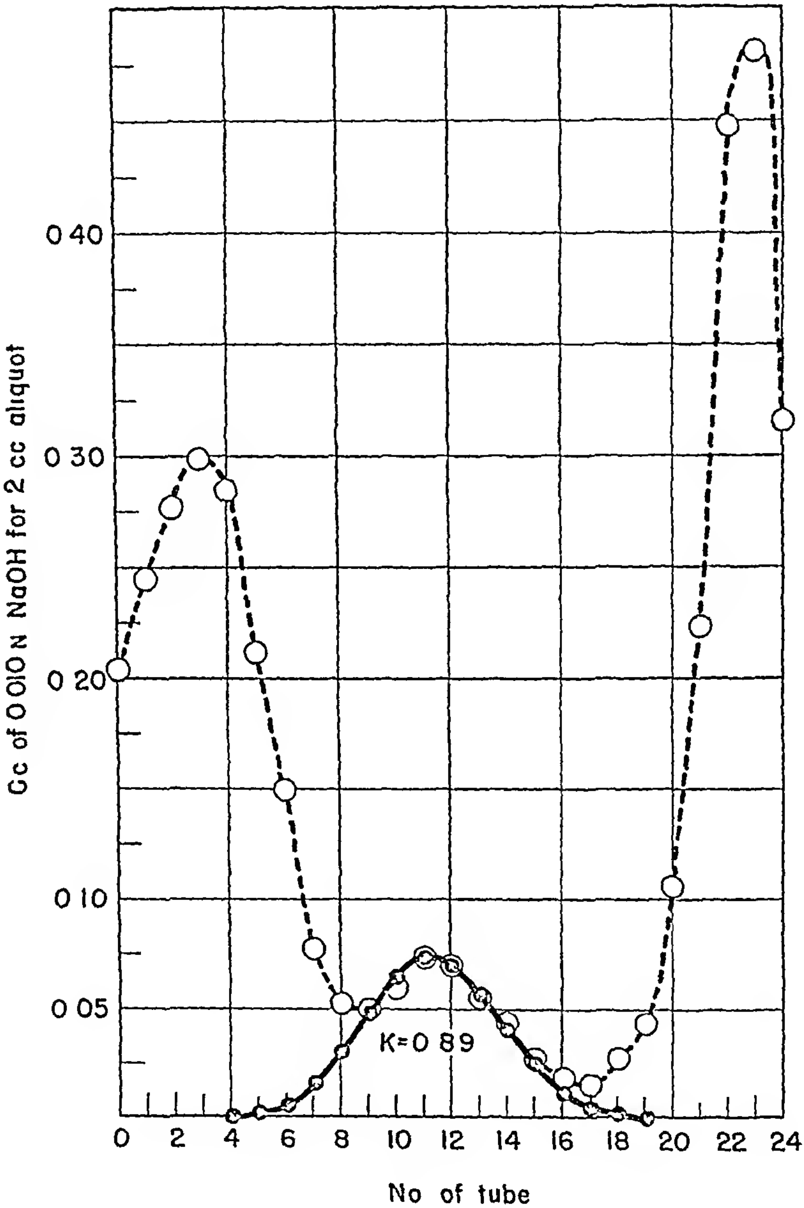


FIG 2 Oxidation of isocaproic acid Solvent system, isopropyl ether and 2 2 M phosphate buffer of pH 5 77 The titration values are for 2 cc aliquot of the initial extraction 300 micromoles of isocaproic acid were present originally, 67 recovered at the finish The *isobutyric peak represents about 18* micromoles O, observed values, ◐, calculated values for *K* of 0 89, as indicated

seemed rather to be the resultant of the action of the enzyme on a substrate which either had been added to the reaction mixture or was originally

present in the enzyme suspension itself In the studies presented here, no attempt has been made to include acetic acid in any type of balance study

Oxidation of Isocaproic Acid—When isocaproic acid was oxidized by the kidney enzyme system and the fatty acids present in the reaction mixture at the end of the experiment were analyzed, the results shown in Fig 2 were obtained The curve with its peak in Tube 11 can be identified as that of isobutyric acid, for it can be seen that its values fit closely the theoretical values calculated for a pure substance with a distribution coefficient of 0 89, which previous tests outside the machine had shown to be the K value for pure isobutyric acid

The distribution coefficient of the substance responsible for the curve at the extreme right of the figure can be shown to be about 14 This curve must represent the remainder of the isocaproic acid originally added, the K for this compound having been shown to be 16 in this solvent pair

The blank for this experiment showed no evidence of the presence of isobutyric acid It was prepared identically with the experimental run, save that the fatty acid substrate was not added until after the incubation period and just prior to deproteinization

This accumulation of isobutyric acid as the result of the oxidation of isocaproic acid must have come about by the splitting off of a 2-carbon fragment from the parent fatty acid, since isovaleric acid did not give rise to isobutyric acid under the same experimental conditions

Oxidation of Isobutyric Acid to Propionic Acid—An examination of Fig 2, with the analysis of the fatty acids arising from the oxidation of isocaproic acid, shows a curve at the far left which appears to be made up of two separate curves For this reason, Tubes 0 to 16 were pooled and the fatty acids contained therein were isolated and redistributed between isopropyl ether and phosphate buffer of pH 5 17 instead of 5 77 (Fig 3) This adjustment of the pH serves to move the peak representing isobutyric acid to the right to Tubes 17 and 18 The values of this new curve fit closely the theoretical curve calculated for a K of 2 6, while that for isobutyric acid in the same solvent pair has been shown to be 2 5

The original composite curve has been separated by this maneuver into two curves The one at the left indicates the presence of acetic acid as usual, while that with its peak in Tube 8 represents an unsuspected substance whose observed values lie closest to the theoretical values calculated for a substance with a K of 0 51 This was taken as presumptive evidence that the unsuspected substance was propionic acid, which was shown to have a K of 0 50 in this system

It was next shown that isobutyric acid on enzymatic oxidation gave rise to this same substance The distribution curve for one of these experiments is shown in Fig 4 Here, although a full set of twenty-four transfers has been made, only Tubes 2 to 13 were titrated The observed values can

be seen to lie very close to the theoretical curve calculated for a K of 0 52 After distribution the contents of Tubes 5 to 12 were pooled and the fatty acid separated, and, when a 0 05 M solution was tested in the lanthanum nitrate-iodine reaction, the result was strongly positive, thus establishing even more firmly that this compound was propionic acid Other possible oxidation products of isobutyric acid were examined to determine whether they were similar to the substance uncovered by counter-current distribution These were methylmalonic acid, β-hydroxyisobutyric acid, α-methylacrylic acid, and α-hydroxyisobutyric acid The first two of these could

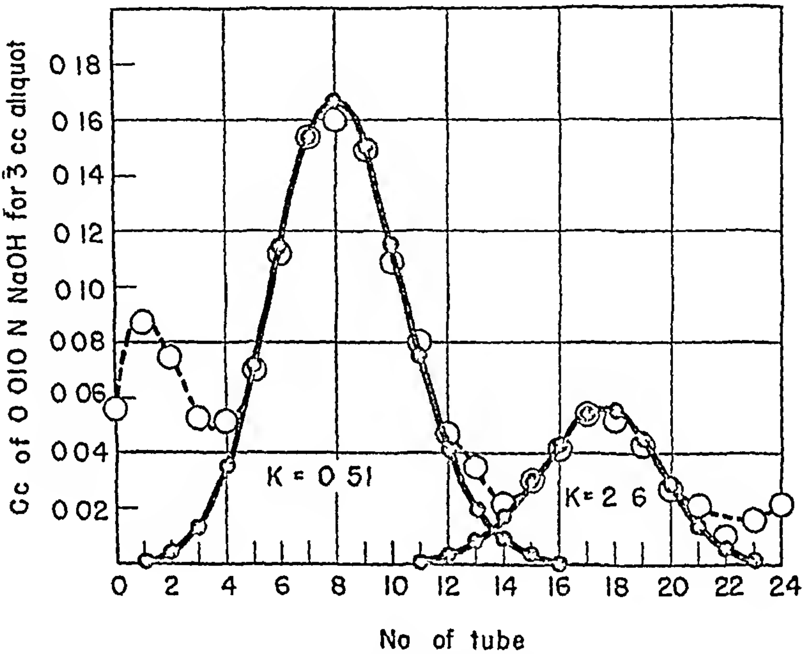
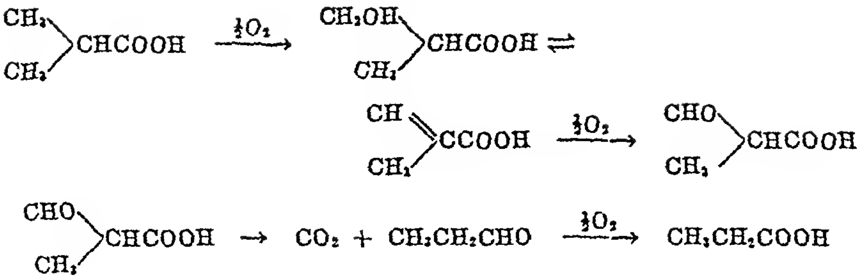


FIG 3 Redistribution of the acid in Tubes 0 to 16 of Fig 2 Solvent system, isopropyl ether and 2 2 M phosphate buffer of pH 5 17 The titration values are for 3 cc aliquot of the initial extraction O, *observed*, o, *calculated for* K *of* 0 51 *and* 2 6, as indicated

be ruled out because they were not volatile under the conditions of the distillation for the experimental run, while the last two could be ruled out on the basis of their distribution coefficients, which were 1 9 and 0 01 respectively in the solvent pair, the aqueous portion of which was phosphate buffer of pH 5 17

Mechanism of Isobutyric Acid Oxidation—Assuming that the oxidation of isobutyric acid to propionic acid involves preliminary β oxidation, the accompanying sequence of reactions may be postulated The liver fatty acid oxidizing system has proved invaluable for demonstrating this mechanism by virtue of its ability to oxidize propionic acid to completion Thus



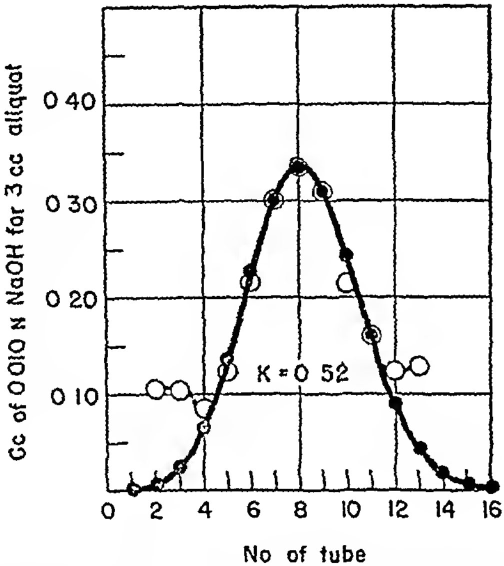


FIG 4 Oxidation of isobutyric acid A twenty four transfer distribution of volatile acids from the final reaction mixture was performed, but only Tubes 2 to 13 were titrated The solvent system was the same as in Fig 3 The titration values are for 3 cc aliquot of the initial extraction 400 micromoles of isobutyrate were added, and 77 micromoles of propionate recovered O, observed, ⊙, calculated for K of 0 52

any substance which is an intermediate between isobutyric acid and propionic acid should be oxidized to completion in the liver system Both β-hydroxyisobutyric acid and methylacrylic acid adequately satisfy that requirement The unresolved point is whether methylmalonic semialdehyde decomposes to propionaldehyde and carbon dioxide or undergoes further oxidation to methylmalonic acid with subsequent formation of propionic acid by decarboxylation Under the conditions of the experiment, methylmalonic acid appeared completely inactive Thus the pathway through propionaldehyde is indicated On structural grounds there is reason to anticipate that methylmalonic semialdehyde would decompose

rapidly to propionaldehyde. Consistent with this prediction is the fact that propionaldehyde can undergo extensive oxidation in the liver fatty acid oxidizing system, while only partial oxidation takes place in the kidney system.

EXPERIMENTAL

Enzyme System—In all cases the enzyme used was the triply washed residue of the rabbit kidney homogenate prepared as previously described (6). In its final form this consisted of a thick suspension in 0.9 per cent potassium chloride.

The experimental runs were set up on a scale 20 to 40 times that of the amounts used in a single Warburg cup. A pilot run was always set up to determine the activity of the particular enzyme preparation used and to provide information on the course of the reaction. 1.5 cc. of the enzyme suspension were used in each Warburg cup and this was supplemented by 0.3 cc. of 0.125 M phosphate buffer of pH 7.3, 0.3 cc. of 0.01 M adenosine triphosphate or adenosine monophosphate, and 0.2 cc. of 0.02 M magnesium sulfate. Alkali-soaked filter paper was placed in the center well. In the studies of the oxidation of valeric and isocaproic acids, 10 micromoles of fatty acid substrate were added to each Warburg cup, while 5 micromoles of α-ketoglutaric acid were added as a cooxidant. In the study of isobutyric acid oxidation, 10 micromoles of the cooxidant were added to an equivalent amount of the fatty acid. All substrates were added in the form of their neutral sodium salts. The large scale experiments were carried out in macro manometer cups with oxygen as the gas phase and at 38°.

Preparation of Samples for Distribution—After appropriate incubation periods, the reaction mixtures were removed from the bath and precipitated by the addition of 2.0 cc. of 10 N sulfuric acid and 7.5 cc. of 0.4 M sodium tungstate solution for every 10 cc. of the reaction mixture. In the studies with valeric and isocaproic acids, the fatty acids were separated from the suspension by steam distillation and the distillate was then subjected to another purification distillation after the addition of 2.0 cc. of 10 N sulfuric acid, 50 gm. of magnesium sulfate, and about 0.2 gm. of mercuric oxide. In all the other experiments the original tungstate precipitate was filtered off, the residue washed once, and the combined filtrates then subjected to the purification distillation. As a final step the distillates were neutralized with sodium hydroxide and evaporated to dryness.

Counter-Current Distribution—The machine was the stainless steel type described by Craig (3). In each tube the aqueous phase consisted of 8.0 cc. of 2.2 M phosphate buffer of pH 5.17, 5.19, or 5.88, while the organic phase consisted of 8.0 cc. of isopropyl ether. The sample to be analyzed was taken up in 8.0 cc. of the buffer and added to Tube 0. After twenty-

four transfers, the contents of each tube were acidified by the addition of 1 0 cc of 8 N phosphoric acid and the amount of acid in the ether phase of each estimated by titration of a suitable aliquot with 0 010 N sodium hydroxide in a micro burette

It is impossible to drive all the fatty acid in each tube into the ether layer for titration by the addition of phosphoric acid as described Therefore, when it was desired to know the total amount of acid in any tube, it was necessary to extract the buffer layer a second time with 8 0 cc of ether and again titrate a suitable aliquot The total amount of acid C can then be calculated from the formula $C = A^2/A-B$ where A is the value for the first titration figure and B is the value for the second (7)

At any point on the curve plotted from the above titration values, the distribution coefficient of the substance responsible for the curve could be determined by the formula

$$K = T_r + 1/T_r \times (r+1)/(n-r)$$

in which K is the distribution coefficient, r is the number of the tube, T is the titration value for that tube, and n is the number of transfers (in these experiments, always twenty-four)

SUMMARY

Valeric acid and isocaproic acid are oxidized in the kidney fatty acid oxidizing system to propionic acid and isobutyric acid respectively Part of the isobutyric acid formed is further converted to propionic acid The end-products were identified by counter-current distribution

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FURTHER STUDIES ON A FAT-SOLUBLE MATERIAL FROM PLASMA HAVING BIOTIN ACTIVITY

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A fat-soluble material from plasma has been found to have biotin-like activity for chicks as well as for lactic acid bacteria (1) Since oleic acid can replace biotin in the nutrition of *Lactobacillus casei* (2, 3) and of a variety of related bacteria (4) as well as in that of a yeast (5), it was of interest to determine whether oleic acid might also have a biotin-like activity for chicks It is the purpose of this paper to report experiments concerned with this problem and to provide additional data on the biotin activity of the fat-soluble material from plasma, and on its occurrence and partial purification

EXPERIMENTAL

General Methods—Biotin activity was determined by microbiological assay with *Lactobacillus casei* with the method of Landy and Dicken (6) slightly modified (1) Fat-soluble materials to be assayed were dissolved in warm ethyl alcohol, and distilled water was added to the solution to give a uniform emulsion

The crude fat-soluble biotin-active material (FSF) was prepared in quantity by the ether extraction of acid-hydrolyzed horse plasma (1) 20 to 30 ml of a neutral dark brown oil were ordinarily obtained from 10 liters of oxalated plasma The activity of such preparations ranged from 1 5 to 3 γ of biotin activity per ml, and was usually about 2 γ

All the chicks used were Rhode Island reds obtained when 1 day old They were kept in electrically heated brooders until 10 to 14 days old, and then in wire bottom cages in a warm room The stock diet was a chick starting mash of the following composition yellow corn-meal 29, ground wheat 29, soy bean meal 17, alfalfa leaf meal 7 5, meat scrap 4 5, cod liver oil 0 7, charcoal 0 7, salt 0 7, calcite 2 1, fine grit 4 4, fine oyster shell 4 4 It contained about 13 γ of biotin per 100 gm of diet For the production of biotin deficiency, this mash was mixed with egg white, usually in the proportion of 20 parts of a commercially dried egg albumin in 100 parts of diet A control casein diet contained in place of egg white washed casein mixed with riboflavin to provide 5 mg of riboflavin per 100 gm of casein

The chicks were kept on the stock diet until they were 5 to 7 days old

They were then placed on the experimental diets and the treatments, if any, begun During the period of the experiment each chick received once weekly 3 to 4 drops of haliver oil with viosterol The treatments consisted usually of the intramuscular injection of biotin, oleic acid, or FSF The injections were given as 0 2 ml of liquid in the breast muscle two times per week The biotin was dissolved at appropriate concentrations in 0 85 per cent sodium chloride solution The FSF and oleic acid were injected as the warm oils themselves FSF was absorbed much better than oleic acid, but with both materials abscesses formed in the breast muscle surrounding small pockets of the oil

At suitable intervals the experimental chicks were weighed and the severity of the dermatitis on the feet and at the corners of the mouth graded by a method similar to that of Ott (7), except that the scale used ranged from 0 to 6 Only occasional chicks, however, showed a dermatitis more severe than grade 4 In order to make this grading as nearly objective as possible, the chicks were taken at random from the various groups by an assistant and presented to the person doing the grading with their number tags covered The latter individual then gave his judgment of the severity of the dermatitis to the assistant, who noted it beside the appropriate number Thus the person grading the chicks could not be aware of the nature of the results until all the chicks had been graded This precaution was desirable, since the experiments with FSF could not be performed in such a way as to give any likelihood of complete prevention of biotin deficiency symptoms The maximal dosage of FSF which could be administered by injection was far from sufficient, in terms of its microbiological biotin activity, to be expected to provide complete protection from the deficiency For this reason most of the experiments included two control groups injected with biotin, one with a dosage similar, on an activity basis, to that of the group receiving FSF and one with a dosage adequate (8) to prevent the deficiency entirely Larger amounts of FSF could be administered by stomach tube, but it has not so far been possible to find any activity of the material when given by this route to chicks on an egg white diet

Results

Treatment of Chicks on Egg White Diet with FSF and Oleic Acid—The effect previously reported (1) of FSF in mitigating the dermatitis produced in chicks by a diet high in egg white has been repeatedly confirmed Tables I and II give the results of two experiments of this type, the usual commercial preparation of egg white having been used for one experiment and an acetone-precipitated preparation from fresh egg white (9) for the other In Table I it will be noted that neither the group receiving FSF nor the groups receiving the lower dosage of biotin gained weight to any greater

extent than the untreated group on the egg white diet Since this was also true in all the other experiments, the weights are not included in Tables II to V It has been found by others (10) that lower dosages of biotin are required to affect the dermatitis than to permit normal weight gain in chicks

Preliminary experiments with oleic acid revealed no effect on the dermatitis of chicks on an egg white diet The results of two experiments com-

TABLE I

Effects of FSF and Biotin on Biotin Deficiency in Chicks Fed Diet High in Egg White

The chicks were started on the special diets and injections when 1 week old

Diet	Injection (breast muscle)	Weekly dosage microbiological assay	No of chicks	Average weight (gm)					Average degree of dermatitis of feet and mouth		
				7 days	15 days	21 days	28 days	39 days	21 days	28 days	39 days
		γ biotin		gm	gm	gm	gm	gm			
Casein, 20%	None		7	57	98	156	203	281	0	0	0
Egg white, 20%	"		7	58	87	133	163	205	3 4	3 3	4 9
	FSF	0 8	7	58	85	125	150	182	1 7	2 7	3 0
	Biotin	0 8	7	57	96	138	159	205	0 7	0 4	1 6
	"	6 0	6	55	88	140	182	251	0 7	0	0

TABLE II

Effect of Intramuscular Injection of FSF in Chicks on Diet containing 500 Gm of Dried Acetone Precipitated Fresh Egg White per 20 Pounds of Feed

Chicks placed on diet when 8 days old, injections begun 3 days later

Treatment	No of chicks	Average degree of dermatitis of feet and mouth	
		21 days	28 days
None	8	1 5	2 1
FSF, 0 8γ biotin activity per wk	9	1 0	1 5

paring FSF preparations with U S P oleic acid and with a soy bean oil distillate (SBO) containing oleic acid[1] are shown in Tables III and IV Although the oleic acid and the SBO had higher biotin activities for *Lactobacillus casei* than the FSF preparations, neither reduced the extent of the dermatitis, while both FSF preparations did so, one of them (from horse plasma, Table IV) to almost as great an extent as did a comparable dosage of biotin

A single somewhat purified preparation of FSF has been tested in chicks

[1] The molecular distillate of soy bean oil was very kindly supplied by Dr P L Harris of Distillation Products, Inc

and found to be active (Table V) Table V also shows that FSF is active when injected into the posterior peritoneal cavity as well as when injected intramuscularly

TABLE III

Intramuscular Injection of U S P Oleic Acid, FSF from Human Plasma Fraction IV-1, and Biotin in Chicks Placed on Egg White Diet When 6 Days Old*

Injections begun at 7 days of age

Injection	Weekly dosage, by microbiological assay	No of chicks	Average degree of dermatitis of feet and mouth			
			20 days	30 days	34 days	44 days
	γ biotin					
None	0	7	1 2	3 2	2 9	3 2
Oleic acid	1 2	8	1 3	2 7	2 9	3 6
FSF	0 6	8	1 0	2 8	2 2	2 6
Biotin	0 6	7	1 1	1 7	1 6	1 1
"	6 0	8	0 8	0 7	0 5	0 1

* A large supply of Fraction IV-1 from human plasma was obtained through the generosity of the American Red Cross

TABLE IV

Comparison of Effects of Intramuscular Injection of Soy Bean Oil Distillate (SBO) containing Oleic Acid, of FSF from Horse Plasma, and of Biotin in Chicks on Egg White Diet

Diet and injections begun at 5 days of age

Injection	Weekly dosage, by microbiological assay	Average degree of dermatitis of feet and mouth (10 chicks)		
		21 days	28 days	34 days
	γ biotin			
None	0	1 8	2 6	2 9
SBO	1 2	2 0	2 4	2 6
FSF	0 9	1 5	1 7	2 0
Biotin	1 0	1 4	1 3	1 6
"	10 0	0 4	0 1	0 2

Plasma and Tissue Levels of Biotin and Fat-Soluble Biotin-Active Materials—The plasma levels of biotin and FSF in young ducks and chickens and the changes which they undergo during acute infections with avian malaria parasites have been previously described (11) The biotin content of the plasma of normal young ducks and chickens is about 2 to 4 mγ per ml, while the bound FSF content in terms of its biotin activity is about 10 to 15 mγ per ml No bound biotin and relatively little free FSF have been found in the plasma

Assays of the plasma of adult birds have shown that, while in males both the biotin and FSF remain much the same as in young birds, in females

TABLE V

Effects of Injection of FSF from Horse Plasma (Fraction A), of Fraction F Obtained from It by Adsorption on Aluminum Oxide and Elution with Ammoniacal Alcohol, and of Biotin in Chicks on Egg White Diet

Diet and injections begun at 5 days of age

Injection	Weekly dosage by microbiological assay	No of chicks	Average degree of dermatitis of feet and mouth	
			19 days	26 days
	γ biotin			
None	0	5	1 7	2 8
Fraction F*	3 0	7	1 2	2 1
" A*	1 2	6	1 3	2 2
" A†	1 2	5	1 4	1 8
Biotin*	1 0	7	0 9	1 5
" *	10 0	6	0 6	0 4

* Injected to breast muscle

† Injected to posterior peritoneal cavity

TABLE VI

Biotin and FSF Content of Plasma of Adult Ducks (5 to 6 Months Old) As Determined in Two Different Ways

Duck		Biotin activity mγ per ml plasma					
No	Sex	Free biotin		Bound biotin	Free FSF	Bound FSF	
		(a)	(b)	(c)	(d)	(e)	(f)
1	♀	23 3	29 2	0	2 2	34 0	28.1
2	♀	26 0	29 3	−1 3	3 2	38 8	35 5
3	♀	30 0	30 0	−2 5	4 5	31 5	31 5
4	♀	32 0	33 0	−1 8	1 5	27 8	26 8
5	♂	4 7	6 3	−1 7	1 1	17 7	16 1

(a) The activity of plasma diluted in water and shaken with ether (b) The activity of plasma hydrolyzed in sulfuric acid minus the activity of the same preparation tested in the presence of 1 500 egg white (c) The activity of plasma hydrolyzed in sulfuric acid and shaken with ether minus the free biotin (a) (d) The activity of plasma diluted in water minus the free biotin (a) (e) The activity of plasma hydrolyzed in sulfuric acid minus the activity of plasma diluted in water (f) The activity of plasma hydrolyzed in sulfuric acid and tested in the presence of 1 500 egg white, minus the free FSF

which are laying eggs both values are increased Table VI gives a small representative sample of the data which have been obtained and at the

same time shows the good agreement between the values as determined by removal of FSF with ether and by inactivation of the biotin with egg white Egg-laying hens showed a high biotin and FSF content of the plasma entirely similar to that of the egg-laying ducks The increase in biotin may well be related to the high biotin content of the yolk of the egg, which in turn is essential for hatchability (12) In a similar way, avidin secretion by the oviduct accompanies egg-laying activity in hens (13) An increase in the total plasma lipides of egg-laying chickens has been demonstrated (14) Evidently the lipide or lipides with biotin activity share in this increase It is noteworthy that the free FSF activity remains relatively low in the plasma of the egg-laying birds, most of the increase occurring in the FSF which is bound, presumably as a lipoprotein

Much of this bound FSF can be precipitated by dilution of the plasma of egg-laying hens or ducks with distilled water in a ratio of 1 20 The protein so precipitated and collected by centrifugation redissolves readily in phosphate buffer of pH 7 4 to give a highly opalescent yellow solution The bound FSF activity of such solutions, expressed as millimicrograms of biotin per mg of protein, was 1½ to 3 times as high as that of the original plasma

Some determinations have been made of the distribution of biotin and fat-soluble biotin-active materials in tissues other than blood The results for 5 to 6 month-old chickens are presented in Table VII Since in the growth of *Lactobacillus casei* biotin can be replaced by oleic acid and a variety of related compounds, the microbiological assay of biotin activity due to fat-soluble materials from tissue would determine the sum of the concentration in the tissue of a number of substances That oleic acid in depot fats did not enter appreciably into the measurements is indicated by the very low FSF activity of the abdominal fat, a tissue which contains about 40 per cent by weight of oleic acid combined as glycerides (15) Whatever the actual substances from tissues may be which have here been measured as FSF, it is remarkable that, except for the liver and kidney which have exceptionally high biotin contents, there is a general parallelism between the biotin content and the relative concentration of the fat-soluble biotin-active materials A similar situation exists in egg yolk For example, the yolk of one egg had a total biotin activity of 750 mγ per ml, of which 360 mγ remained after shaking with ether The figures for biotin proper which are here presented are for the most part somewhat lower than those previously reported, though of the same order of magnitude Thus Eakin *et al* (16) found for the organs of a 10 week-old chicken 2600 mγ per gm in the liver and 2500 mγ in the kidney, but only 65 mγ in the brain Values for egg yolk have been found ranging from 400 to 600 mγ per ml (17) with a usual value of about 500 mγ per gm (12)

Partial Purification of FSF from Horse Plasma—Two different methods have yielded products having as high a specific activity (millimicrograms

TABLE VII

Biotin and FSF Contents of Representative Tissues from 5 to 6 Month-Old Chickens

Samples of the tissues were ground in saline in a glass grinder, autoclaved in 2 5 N sulfuric acid for 1 hour at 15 pounds, brought to pH 9 6, and diluted with water The uniform suspensions thus obtained were divided into two portions, one of which was assayed for its total biotin content, while the other was shaken with three to four portions of ether and then assayed The results of the latter assay gave the biotin content of the preparation, while the difference between the two assays was considered as activity due to FSF In some cases, indicated by an asterisk, FSF activity was determined directly by the assay of suitable dilutions of the suspension in the presence of egg white

The results are expressed as millimicrograms of biotin activity per gm of fresh tissue

Sex	Chicken No	Brain	Lymph nodes	Breast muscle	Liver	Abdominal fat	Spleen	Oviduct	Testis	Adrenal glands	Kidney
♀	1	87†	34	13	592	7	45	80			993
		(165)‡	(83)	(43)	(129)*	(10)	(83)	(82)			(122)*
	2	107	57	15	1380	2	72	134			1390
		(203)	(105)	(52)	(160)*	(4)	(98)	(106)			(110)*
	3	97	38	31	780	6	55	179		172	1790
		(195)	(77)	(40)	(131)*	(5)	(116)	(88)		(248)	(74)*
	4	69	37	13	750	4	52	53		112	805
		(160)	(75)	(35)	(102)*	(4)	(82)	(99)		(151)	(60)*
	5	142	49	29	1250	8	68	123			
		(141)	(60)	(48)	(75)	(15)	(102)	(79)			
	6	127	87	36	740	3	77	118		131	1150
		(191)	(106)	(41)		(2)	(89)	(61)		(133)	
♂	7	91	46	19	850	15	42			105	895
		(168)	(65)	(35)	(62)*	(8)	(103)			(140)	(56)*
	8	87	187	10	605		38			150	1675
		(174)	(58)	(43)	(64)*		(99)			(136)*	(72)*
	9	126	80	27	1220	13	64			170	2140
		(189)	(101)	(39)		(17)	(110)			(142)	
	10	82	44	18	1210	5	42			211	1625
		(228)	(76)	(34)	(60)	(4)	(88)				(0)
	11		197	24	1040		91		66	86	1123
			(100)	(42)	(180)		(71)		(114)	(151)	(162)
Averages		101	78	21	947	7	59	114		142	1369
		(181)	(83)	(41)	(107)	(7)	(95)	(86)		(157)	(82)

† The upper figures represent biotin throughout

‡ The lower figures represent FSF throughout

of microbiological biotin activity per mg of dry weight) as commercial samples of U S P or C P oleic acid The products so obtained differed

obviously from oleic acid in being at ordinary room temperature (20–25°) whitish or pale yellow waxy solids

One method was a crude counter-current distribution employing nine separatory funnels (18) Table VIII illustrates the results of such an experiment The starting material in this case was prepared by fractionation in alcohol 75 ml of the oil from hydrolyzed horse plasma were suspended in 200 ml of hot alcohol. The insoluble portion was extracted with two more portions of hot alcohol. The combined alcohol solutions were refrigerated overnight and filtered in the cold until clear The portion soluble in cold alcohol, which contained virtually all of the activity, was concentrated *in vacuo* to remove the alcohol A dark brown oil was obtained

TABLE VIII

Counter-Current Distribution of FSF Activity in Nine Separatory Funnels between Cyclohexane and 90 per Cent Alcohol

Funnel No	Total activity for *Lactobacillus casei* when tested at concentrations of 1 2000 and less	Specific activity
	mγ	*mγ per mg dry weight*
0	0	
1	420	
2	900	4 1
3	880	4 0
4	820	5 1
5	540	6 8
6	360	
7	60	
8	0	
Total recovered out of 3750 mγ	3980	

which solidified in the cold 1 ml of the oil was dissolved in cyclohexane (previously shaken with 90 per cent ethyl alcohol) to a volume of 10 ml This solution was then used for an eight plate distribution between cyclohexane and 90 per cent ethyl alcohol, each of which had been previously thoroughly shaken with the other solvent A theoretical curve based on the distribution coefficient (about 1 1) of the biotin activity between cyclohexane and 90 per cent alcohol would have placed the maximal amount of activity in Funnel 4 Actually, it was distributed between Funnels 2 to 4 However, the highest specific activity occurred in Funnel 5 In another similar experiment the maximal amount of activity was distributed between Funnels 3 to 5 and the specific activity of the material from Funnel 4 was 10 mγ per mg A sample of U S P oleic acid dissolved in alcohol, which was assayed at the same time with this fraction and for which the dry weight

was determined in the same manner, gave a specific activity of 10 9 mγ per mg However, in other assays the specific activity of oleic acid would be as low as 7 mγ per mg It is important to note here that some fluctuation in the assay results is introduced as a result of the toxicity of higher concentrations of the fat-soluble materials The toxic effect was relatively small with most of the preparations of the original oil from hydrolyzed horse plasma, so that at suitable concentrations nearly maximal growth was obtained Moreover, with these crude FSF preparations the dose-response curve closely approached that with biotin itself (1) Such is not the case with oleic acid (2) or with the more highly purified preparations of FSF Higher concentrations of these substances all exhibit a growth-inhibiting effect on *Lactobacillus casei*, before maximal growth has been attained

TABLE IX

Chromatographic Adsorption of FSF from Alcoholic Solution onto Aluminum Oxide

Solvent passing through column	Fraction No (each collected from bottom of column for 10 min period)	Total biotin activity for *L casei*	Specific activity
		mγ	*mγ per mg dry weight*
95% ethyl alcohol	1,2	0	
	3	10	
	4–8	0	
5% ammonia in 66% ethyl alcohol	9–12	0	
	13	15	
	14	45	9 0
	15	70	10 8
	16	60	9 2
Starting material		1920	3 2

The second method of partial purification depended on chromatographic adsorption on aluminum oxide and elution with ammoniacal alcohol The activity could be adsorbed either from a cyclohexane solution or from an alcoholic solution containing only that portion of the original oil which was soluble in cold alcohol When such a solution was allowed to percolate through a 10 × 2 cm column of aluminum oxide prepared in the corresponding solvent and was followed by more of the same solvent, an orange-yellow zone soon formed and migrated down the column at a fairly rapid rate All the percolates appeared colorless, except those which included the zone and which were bright yellow In repeated trials, the fraction containing the colored material has had a low but significant activity, whereas the fractions preceding and following it have had little or no activity Most of the activity of the original solution, however, remained ad-

sorbed as long as cyclohexane, 95 per cent alcohol, or alcohol acidified with HCl was passed through the column Elution began soon after the addition of ammoniacal alcohol (5 per cent ammonia in 66 per cent ethyl alcohol) and continued slowly over a considerable period Tables IX and X give the results of two experiments of this type In the experiment illustrated in Table IX, the yellow material was collected as a separate fraction (No 3) It is obvious that in this experiment elution was still occurring at an appreciable rate when the collection of fractions was discontinued The high specific activity of Fractions 14 to 16 is worthy of note In the larger scale experiment illustrated in Table X the yellow material was not collected separately but it probably was responsible for most of the 460 mγ of activity

TABLE X

Chromatographic Adsorption of FSF from Alcoholic Solution onto Aluminum Oxide

Material	Total activity	Specific activity
	mγ	*mγ per mg dry weight*
Starting material Cold alcohol-soluble portion of oil from hydrolyzed horse plasma in 95% alcohol	20,000	
Combined percolates from sample and 100 ml 95% alcohol	460	
1st hr 's percolate after start of 5% NH_3 in 66% alcohol	270	
Next 20 min percolate	1,000	3 2
" 20 " "	1,000	5 4
" 20 " "	800	
" 20 " "	800	6 7
" 20 " "	800	6 7
Total recovered in percolates	5,130	

present in the combined percolate from the sample and the following 100 ml of alcohol

A larger quantity of a partially purified material was prepared by gross adsorption and elution 95 ml of the oil from horse plasma were dissolved in cyclohexane to 400 ml 150 gm of aluminum oxide were added and the mixture was stirred for half an hour It was then filtered To the filtrate were added an additional 100 gm of aluminum oxide, and the mixture was again stirred for half an hour It was filtered through the same paper which had been used previously, and the combined residues were washed with 300 ml of cyclohexane The combined filtrates were concentrated *in vacuo* at 45–50° to give 50 ml of a brown oil with about one-fourth the activity of the starting material The aluminum oxide residue was sus-

pended in 560 ml of 5 per cent ammonia in 66 per cent ethyl alcohol and shaken mechanically half an hour The aluminum oxide was allowed to settle and the supernatant was poured off through a filter paper The aluminum oxide was then resuspended in an additional 280 ml of ammoniacal alcohol and was again shaken mechanically for half an hour The mixture was filtered The combined clear amber filtrates were brought to a pH of 7 with 6 N HCl to give a cloudy liquid with droplets of brown oil This was shaken in 100 ml portions with 100 ml of cyclohexane in a separatory funnel The same cyclohexane was used for three portions of the neutralized eluate All of the aqueous-alcoholic lower layers were pooled On concentration of these *in vacuo* at 50° droplets of brown oil appeared when the volume was about 200 ml This material was taken up in ether On removal of the ether there were obtained 4 ml of dark brown oil with an activity of 6 5 γ per ml The combined cyclohexane extracts were concentrated *in vacuo* at room temperature to a small volume and left overnight A light brown waxy material was obtained which, when warmed, became an easily flowing light brown liquid 12 ml of this material were obtained, with a biotin activity for *Lactobacillus casei* of 7 6 γ per ml This was the fraction (F) which was found to have biotin-like activity for chicks (see Table V)

DISCUSSION

The experiments reported in the present paper support the conclusion that hydrolyzed plasma yields a substance, readily extractable with ether, which, when injected intramuscularly, has biotin-like activity in reducing the extent of the dermatitis produced in young chicks by a diet high in egg white Usually the effect was not as great as that obtained by the injection of a comparable dosage of biotin in terms of microbiological biotin activity Several explanations for this may be suggested In the first place, while there was never any leakage of the injected saline-biotin solutions on withdrawal of the needle, some leakage of the oils with FSF activity frequently occurred While the saline was rapidly and completely absorbed, this was not true of the oil, which moreover had some toxic properties Finally, it is possible that the oil obtained from hydrolyzed horse plasma contains more than one substance with biotin activity for *Lactobacillus casei* but only one with biotin activity for chickens This possibility is supported by the high biotin activity for *Lactobacillus casei* of oleic acid, a substance with which it has not been possible to demonstrate any biotin activity in chickens

In the studies directed toward purification of the active material in FSF the activity was followed only by microbiological assay, since amounts sufficient for assay in chicks could not readily be obtained It was hoped that

methods in which the material was subjected to relatively mild treatments would yield final products retaining their activity for chickens This hope was realized with the partly purified fraction (F) obtained by adsorption and elution More drastic procedures such as saponification were avoided, although in the first work concerning the fat-soluble biotin-active substance from hydrolyzed plasma it was found that the activity for *Lactobacillus casei* remained in the unsaponified fraction (1). Hofmann and Axelrod (19), in their note confirming the finding of a neutral oil from plasma with biotin activity, reported that the activity went into the saponifiable fraction On the basis of personal communication, it would appear that this discrepancy is the result of a longer period of heating with alkali employed by the latter workers than by the former, who heated for 45 minutes With several hours heating, the activity of FSF for *Lactobacillus casei* goes into the saponifiable fraction Such saponified material has not been tested for its activity in chicks

A number of authors have suggested ways in which oleic acid and related compounds might function in bacterial metabolism Dubos (20) has concluded that in the growth of his *Micrococcus* C they probably act as catalysts, whereas in the growth of tubercle bacilli they enter into the synthesis of cell protoplasm Guirard *et al* (21) have noted their rôle as substitutes for acetate in the nutrition of some of the lactic acid bacteria Still other lactic acid bacteria require oleic acid in addition to both biotin and acetate (4, 22, 23) Williams and Fieger (3) have postulated that both biotin and oleic acid function as cell permeability factors Perhaps somewhat more likely is the hypothesis (4) that biotin is essential for the synthesis of oleic acid as it is for the synthesis of aspartic acid (24, 25)

The latter hypothesis could readily be applied to multicellular organisms, perhaps with the further addition that biotin enters into more than one stage of the synthesis of fatty compounds It was early noted by Boas (26) that rats with severe biotin deficiency had almost no stores of body fat Fatty infiltration of the liver and increase in body fat of rats have been observed after biotin administration (27), and more recently it has been found that rats with incipient biotin deficiency, unlike normal rats, did not store excess lipide in the liver when fed a diet high in cholesterol (28) The activity of the neutral oil from hydrolyzed horse plasma for chickens as compared to the inactivity of oleic acid for these animals suggests that perhaps in vertebrates the fatty acid must be supplied already combined in some manner in order to eliminate partially the need for biotin It is also possible that the neutral oil is effective merely because it provides the fatty acid in less toxic form (4) In this connection it is interesting to note that for a quite different metazoan organism, the larva of the yellow fever mosquito, it has been found that, while both oleic acid and FSF can replace

biotin, the former material is active over a narrower range than the latter, presumably because of its greater toxicity[2]

SUMMARY

The intramuscular injection of a fat-soluble material from hydrolyzed plasma into chicks fed a diet high in egg white reduced the severity of their dermatitis almost as much as did the injection of biotin in a comparable dosage in terms of microbiological biotin activity The similar injection of oleic acid did not have such an effect, nor could the effect be produced by the oral administration of the material from plasma

The concentration of the fat-soluble biotin-active material, as measured by microbiological assay in the plasma of ducks and chickens, shares in the general increase in lipides which occurs with the onset of egg-laying activity The distribution of this material in the tissues of birds roughly parallels the distribution of biotin, except in the liver and kidney, which contain relatively very large amounts of biotin Much of the bound fat-soluble biotin-active material in the plasma of egg-laying hens may be concentrated in a protein fraction precipitated by dilution of the plasma with water

Partial purification of the active material from hydrolyzed horse plasma has been effected by counter-current distribution in separatory funnels and by chromatographic adsorption Fractions have been obtained which have as high a specific activity for *Lactobacillus casei* as oleic acid but which differ from oleic acid in physical properties A somewhat similar fraction prepared by gross adsorption and elution has been shown to have the biotin-like activity when injected into chicks on an egg white diet

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ELECTROMETRIC AND COLORIMETRIC DETERMINATION OF CARBONIC ANHYDRASE

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The activity of carbonic anhydrase has been estimated by three procedures manometric (1–3), colorimetric (2, 4–8), and electrometric (9) For investigations of the enzyme concentration of tissues the colorimetric method has often been used in preference to the manometric technique because of its relative simplicity A disadvantage of the colorimetric method has been the inhibitory effects of the indicator (10) and of the carbonate-bicarbonate buffer, an effect also true of phosphate buffer in the manometric method, though to a lesser degree (8) Roughton and Booth (8) have found that veronal buffer is without noticeable effect on carbonic anhydrase and have suggested a colorimetric method which employs this buffer with brom-thymol blue This method permits the measurement of enzyme activity in terms of moles of CO_2 hydrated, allowance being made for inhibition by the indicator However, the influence of the indicator on the enzyme activity in this system has not been studied

This paper describes a new electrometric method which is here employed for evaluation of the inhibitory effect of indicators on carbonic anhydrase preparations from various sources A modification of the veronal colorimetric method suitable for routine determinations of carbonic anhydrase in tissues is also presented together with data on its reliability

Electrometric Method

In the electrometric method suitable amounts of veronal buffer and saturated CO_2 solution are mixed at 0° to give a displacement of the hydrogen ion concentration from approximately pH 8 to 6 3 in a conveniently measurable period of 100 to 120 seconds for the uncatalyzed reaction The apparatus employed (Fig 1) utilizes two automatic measuring syringes (Becton, Dickinson improved Cornwall syringe No 1250) to force buffer and CO_2 solutions simultaneously into a reaction vessel containing a glass electrode and the enzyme or experimental solutions or both The pH changes may be followed (Fig 2) or the time necessary for the solution to reach the end-point (pH 6 3) determined After the reaction is completed, the reaction vessel is flushed and drained through a distilled water-evacuation system

Veronal Buffer—4.536 gm of sodium barbiturate were dissolved in 950 ml of double distilled water, and barbituric acid was added to bring the pH to 8 15 The amount required varied slightly with the source The solution was then made up to 1 liter in a glass-stoppered flask Precautions were taken to prevent carbon dioxide absorption

Saturated CO_2 Solution—CO_2 from a Dewar flask containing solid CO_2 was bubbled through a gas bubbler into double distilled water at 0° for at least 1 hour before use

Indicators—To study the effect of indicators on the catalyzed and uncatalyzed reactions, powdered brom-thymol blue (Harleco) and powdered

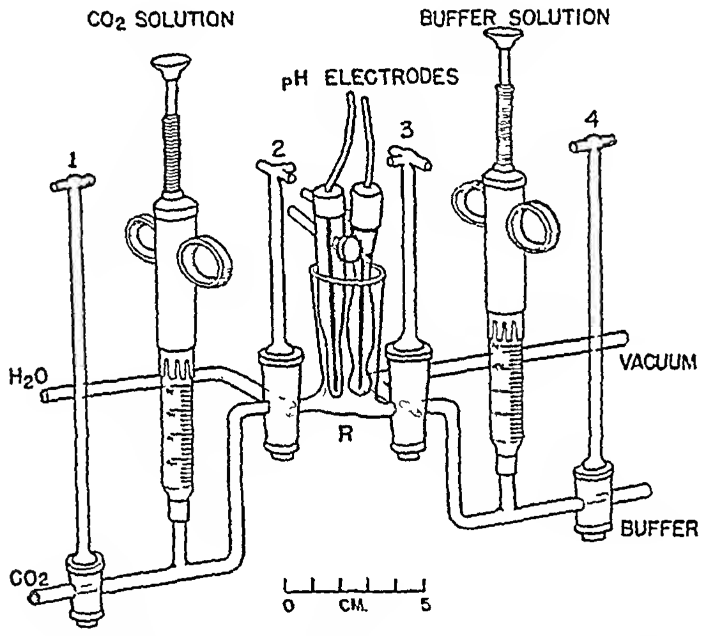

FIG 1 Apparatus for electrometric determination of carbonic anhydrase activity

phenol red (Merck) were made up in double distilled water and adjusted to pH 6 3 with NaOH

Apparatus—Those portions of the apparatus shown in Fig 1 are mounted on a metal support and placed in a glass container, 12 × 7½ × 8¾ inches Connected with the apparatus are (*a*) a 250 ml Mariotte bottle of CO_2-saturated distilled water with a saturating device and a long tube to remove excess CO_2 from the vicinity of the apparatus, (*b*) a 250 ml bottle of veronal buffer with an air vent protected by an NaOH solution, (*c*) a glass cooling coil connected with an elevated distilled water supply, and (*d*) a vacuum line The total assembly was packed in a water-ice mixture in the glass container and placed in a wooden box with glass wool insulation between the two containers It was found convenient to carry out the measure-

ments in a refrigerated room at 4° All experimental solutions were kept at 0°

Standard Beckman electrodes were mounted on a rack and pinion over the reaction vessel and connected through a shielded cable to a Leeds and Northrup pH meter The pH meter and electrodes were standardized in position at 0° with phosphate buffers All solutions used were made with double distilled water

Operation—To place the system in operation, the plunger of the buffer syringe is removed and positive pressure induced through the CO_2 absorption system of the buffer bottle to fill the syringe through Stop-cock 4 (The construction of the stop-cock extensions indicates the orientation of the stop-cock) The syringe is then reassembled and the contents forced into the reaction vessel (*R*) through Stop-cock 3 after Stop-cock 4 is closed With the syringe plunger still down, Stop-cock 3 is turned to neutral position and Stop-cock 4 is opened The slow release of the plunger allows the syringe to fill with fresh buffer Stop-cock 4 is then closed A similar procedure is used to fill the other side of the system with saturated CO_2 solution With care all bubbles can be eliminated from the system

The reaction vessel and electrodes are flushed in the following manner After both sides of the system are filled, Stop-cocks 1 and 4 are closed and Stop-cocks 2 and 3 turned to the positions shown Cold distilled water is allowed to flow into the reaction vessel through Stop-cock 2 until it is full The stop-cock in the vacuum line (not shown) is then opened and the water withdrawn through Stop-cock 3 which is low enough for complete drainage This process is repeated until the vessel and electrodes are clean (see below)

In making a determination all stop-cocks are turned to neutral position 1 0 to 2 0 ml of experimental solution or distilled water are carefully pipetted to the bottom of the reaction vessel Both openings into the reaction vessel are covered by this quantity of solution, so that both the buffer and CO_2 solutions flow in under liquid After temperature equilibrium is reached in this solution Stop-cocks 2 and 3 are turned to connect the reaction vessel with both syringes Both plungers are depressed simultaneously, giving rapid mixing Stop-cocks 2 and 3 are turned at once to neutral position and Stop-cocks 1 and 4 opened Both plungers are then slowly released, refilling the syringes, and Stop-cocks 1 and 4 are then closed

EXPERIMENTAL

Methods—Carbonic anhydrase preparations were made from human blood, gastric mucosa of *Rana pipiens*, and from whole rat brain For blood carbonic anhydrase, hemolyzed erythrocytes and the modified crude chloroform extract of Roughton and Booth (11) were used Neither prep-

aration showed appreciable change in activity when kept in a refrigerator several weeks in dilute solution Mucosal and brain extracts were made by grinding fresh tissue thoroughly and diluting with about 15 volumes of distilled water The suspension was then centrifuged 8 minutes at 850 × g and the supernatant removed and diluted to give a reaction time of 45 to 70 seconds

0 2 ml of enzyme solution was added to 1 0 ml of experimental solution or distilled water in the reaction vessel 30 seconds prior to the addition of 2 0 ml each of CO_2 solution and veronal buffer Contact of enzyme and indicator was limited to this period plus the time of reaction

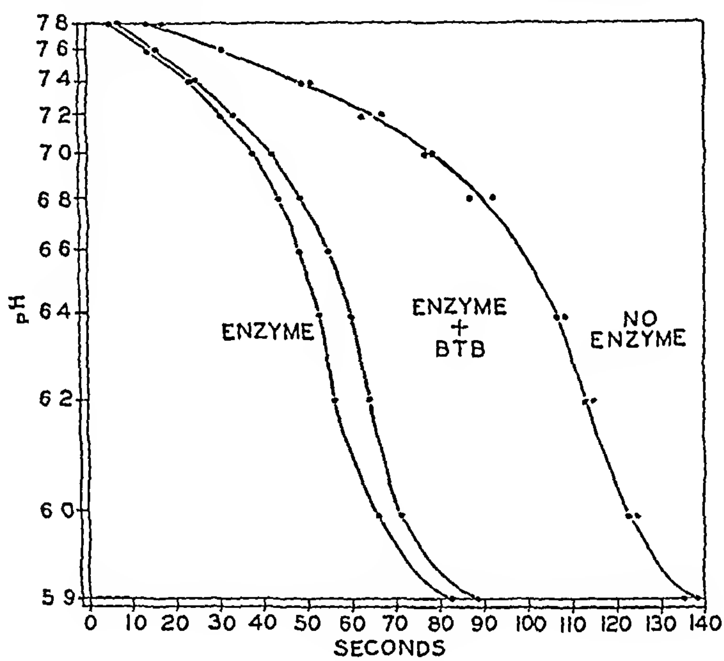

FIG 2 Hydration of carbon dioxide in veronal buffer Displacement of curve of enzyme plus brom-thymol blue indicates slight inhibition

Results—The curves of a catalyzed and an uncatalyzed reaction are shown in Fig 2, duplicate determinations were performed for the uncatalyzed reaction As illustrated by the curves, the rate of change resulting from the low buffering power at pH 6 3 makes this a suitable end-point for both the electrometric method and the colorimetric method of Roughton and Booth (8) with brom-thymol blue

The possibility of enzyme adsorption on the walls of the reaction vessel and the glass electrodes was examined by running a very potent crude blood preparation and then measuring the rate of the uncatalyzed reaction which followed after three and again after six flushings of the reaction vessel Six flushings appear adequate

Brom-thymol blue has an inhibitory action on carbonic anhydrase, the extent depending upon the indicator concentration and the particular enzyme preparation (Table I) With 1 92 mg per cent of indicator, a concentration suitable for colorimetric determinations, the inhibition on rat

TABLE I

Inhibition of Carbonic Anhydrase by Brom thymol Blue

All measurements were made with veronal buffer and electrometric equipment at 0° The pH of the added indicator solution was adjusted at 6 3 The figures for the indicator represent final concentrations Runs with enzyme and enzyme plus indicator were alternated

Enzyme preparation	Indicator	Enzyme + water	Enzyme + indicator	Per cent inhibition	Probability
	mg per cent	*sec*	*sec*		
Rat brain	1 92	53 8	59 5	11	0 001 < P < 0 01
	3 84	48 4	62 9	30	P < 0 001
Frog gastric mucosa	1 92	52 3	56 5	8	0 02 < P < 0 05
	3 84	51 1	62 6	23	0 001 < P < 0 01
Human blood, hemolyzed	1 92	51 2	52 0		0 5 < P < 0 6
	3 84	54 3	60 3	11	0 001 < P < 0 01
Human blood, chloroform extract	1 92	51 7	53 1	3	0 4 < P < 0 5
	3 84	53 8	56 0	4	0 1 < P < 0 2
No enzyme	3 84	113 2	113 7		P < 0 001

TABLE II

Inhibition of Carbonic Anhydrase by Phenol Red

All measurements were made with veronal buffer and electrometric equipment at 0° The added indicator solution was adjusted to pH 6 3 Final concentration of phenol red was 2 88 mg per cent Runs with enzyme and enzyme plus indicator were alternated

Enzyme source	Enzyme + water	Enzyme + indicator	Per cent inhibition	Probability
	sec	*se*		
Rat brain	56 7	57 9		0 7 < P < 0 8
Human blood, hemolyzed	56 5	61 2	8	0 01 < P < 0 02
" " chloroform extract	69 1	71 3	3	0 05 < P < 0 1
No enzyme	115 2	114 1		0 7 < P < 0 8

brain and frog gastric mucosa preparations was about 11 per cent and 8 per cent respectively, with a negligible effect on blood preparations In a similar series with phenol red (Table II) in a concentration of 2 88 mg per cent, as ordinarily used in the Brinkman technique, the indicator was without significant effect except in the case of hemolyzed blood in which there was an 8 per cent inhibition

Colorimetric Method

Apparatus and Procedure—The colorimetric method is an adaptation of that described by Roughton and Booth (8) and utilizes a Cornwall automatic syringe to introduce cold saturated CO_2 solution into a veronal buffer containing brom-thymol blue in a test-tube. The time required for the pH to drop from approximately pH 8 to 6.3 is measured.

The CO_2 saturation system was similar to that used for the electrometric method. In making a determination, 2.0 ml. of saturated CO_2 solution are drawn into a cold syringe, and a small test-tube is placed over a 3 inch No. 20 gage needle and held by a small rubber stopper on the shaft of the needle. The syringe is then immersed in a water-ice mixture until needed. 2.0 ml. of the cold veronal buffer used in the electrometric method containing 5 mg. per cent of brom-thymol blue are then pipetted into a Kimball test-tube, 6 × ¾ inches, with 1.0 ml. of the experimental solution or distilled water, stoppered, and placed in an ice-water mixture. After allowing time for temperature equilibration in the test-tube and the syringe, the syringe is quickly removed from the ice mixture, grasped with a cloth to remove excess water, and the small tube protecting the needle is pulled off. The CO_2 solution is rapidly ejected. With the size of test-tube indicated for the reaction vessel the syringe will come to rest with the tip of the needle immersed near the middle of the buffer solution and centered by the rubber stopper on the needle. After removing the syringe from the test-tube the needle is washed to remove any enzyme before the plunger is released. The needle may be coated with paraffin, though this was not done in the present study. The end-point is determined by matching with a comparator tube containing brom-thymol blue in veronal buffer at pH 6.3. Fogging of the ice bath can be prevented by Anti-Fog, No. 111, supplied by the American Optical Company.

Results—Eleven consecutive determinations of the uncatalyzed reaction gave an average of 103.5 seconds with a probable error of 1.0 second for a single determination. Two series of five runs each with enzyme gave averages of 86.4 and 80.4 seconds, with a probable error of 1.2 and 2.3 seconds for a single determination. Measurements were made with ice-packed equipment in a room at normal temperature.

DISCUSSION

The electrometric method with its provision for the automatic measurement of solutions and washing of the reaction chamber permits the measurement of the carbonic anhydrase activity of a considerable number of samples in a relatively short period of time. While the method has the

disadvantage that the enzyme is subjected to a range of hydrogen ion concentrations during the course of a single measurement, this range may be limited to any desired portion of the curve

The electrometric apparatus may be used without the electrodes in colorimetric determinations by adding indicator to the stock buffer solution, though its chief application is with turbid or colored solutions in which brom-thymol blue cannot be used or in which details of the course of the reaction are required It has been used here to test the effects of indicators on enzymes under conditions which duplicate the colorimetric technique in pH range, indicator concentration, and volume of solutions

The effects of brom-thymol blue and phenol red on different carbonic anhydrase extracts call attention to certain considerations in the use of colorimetric methods Since the extent of inhibition varies with the tissue extract, this value will have to be determined for the individual tissue before corrections can be made in calculations of enzyme activity by the procedure of Roughton and Booth (8) In any case, it is apparent that in the colorimetric method the indicator concentration should be kept as low as is practicable The effects here described relate to the experimental situation in which indicator is mixed with enzyme immediately prior to the addition of the substrate, and may or may not obtain with prolonged contact We are unable to say whether the marked inhibition found by Kiese and Hastings (10) may involve this factor or whether it is due to differences in extract and experimental method

SUMMARY

1 An electrometric technique for the measurement of carbonic anhydrase activity is described In the apparatus employed saturated carbon dioxide and veronal buffer are mixed with automatic measuring syringes and the pH change measured with the glass electrode

2 The electrometric method was employed to study the inhibitory effect of brom-thymol blue and phenol red on the carbonic anhydrase activity of rat brain, frog gastric mucosa, and human erythrocyte preparations, the extent of inhibition was found to vary with the enzyme source and indicator concentration An indicator concentration suitable for use in a colorimetric technique gave an inhibition of 0 to 11 per cent

3 A modification of the Roughton and Booth veronal-brom-thymol blue colorimetric system is described and data on its reliability presented

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THE THYROXINE-LIKE ACTIVITY OF COMPOUNDS STRUCTURALLY RELATED TO THYROXINE*

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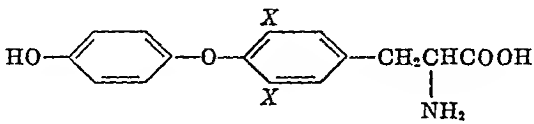
In the course of a search for compounds which might prove to be competitive inhibitors of thyroxine in its action on the peripheral tissues, we have initiated a study of the relationship between chemical structure and thyroxine-like activity of a number of thyroxine analogues

Early attempts to relate chemical constitution to physiological activity have been summarized by Harington (1, 2) who concluded that the structural requirements for activity were best represented by (I), where X is a



(I)

halogen and the alanine side chain may be functionally substituted The report by Loeser and Trikojus (3) of appreciable thyroxine-like activity of thyroxamine, in agreement with the work of Abderhalden *et al* (4) but contrary to that of Gaddum (5, 6), appeared to indicate that an intact alanine side chain may not be required for thyroxine-like activity Later Niemann *et al* (7, 8) suggested the importance of a quinoid resonance involving the phenolic hydroxyl group and the ether oxygen, the amino acid side chain possibly being involved in the incorporation into a peptide linkage These considerations have led us to investigate the effect of other previously untested modifications of the side chain on physiological activity

Materials

DL-Thyroxine[1] was used as the standard in all of the experiments reported It was dissolved in a minimum amount of dilute sodium hydroxide

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† Some of the data were taken from a thesis presented by Earl Frieden to the Graduate School of the University of Southern California in partial fulfillment for the requirements for the degree of Master of Science

[1] We are indebted to Dr Kenneth W Thompson of Roche Organon, Inc , Nutley 10, New Jersey, for a generous supply of DL thyroxine

and the solution adjusted to pH 8 0 ± 0 5 before being made up to volume

3,5-Diiodo-L-tyrosine, m p 202–204°, was prepared from L-tyrosine by the method of Block and Powell (9) The possibility of *in vitro* conversion of diiodotyrosine to thyroxine was minimized in most cases by dissolving a neutral suspension of diiodotyrosine in dilute alkali and adjusting to pH 8 only a few minutes prior to the time of injection

3,5-Diiodo-4-(3′,5′-diiodo-4′-hydroxyphenoxy)-benzoic acid, m p 255°, referred to as the benzoic acid analogue, was obtained as described by Harington and Barger (10) $C_{13}H_6O_4I_4$, calculated, I 69 2 per cent, found, 68 5 per cent It was given as the sodium salt

3,5-Diiodo-4-(3′,5′-diiodo-4′-hydroxyphenoxy)-DL-phenylglycine, the glycine homologue of thyroxine, was synthesized as described by Frieden and Winzler (11) It was administered in the same manner as DL-thyroxine

N-Acetyl-DL-thyroxine, m p 210–214°, was prepared by the method of Kendall and Osterberg (12), with the corrections of Ashley and Harington (13) As expected, the compound gave a negative ninhydrin test and a positive Kendall reaction with nitrous acid It was administered as the sodium salt at pH 8 0 ± 0 5

N-Acetyl-3,5-diiodo-L-tyrosine, m p 198–200°, was synthesized according to Myers (14) This substance also gave a negative ninhydrin test and a positive Kendall reaction with nitrous acid and was administered as the sodium salt

Methods

Amphibian Metamorphosis—The effect of thyroxine and its analogues on amphibian metamorphosis was followed by observing the diminution of total body length essentially according to the method of Gaddum (5) Groups of five larvae (*Bufo* sp) were placed in 250 ml glass dishes containing 200 ml of tap water in which the test substance had previously been dissolved The animals were measured with a micrometer on white oilcloth by stretching them out to their full length In this way, animals could rapidly be classified according to size without injury They were not fed during the course of the experiment, no decrease in length being observed for untreated controls under these conditions Results with at least three different concentrations of DL-thyroxine administered to tadpoles, in addition to untreated controls, were observed along with the test substance in each experiment Experiments were brought to a conclusion when a thyroxine control containing 0 25 γ per ml revealed a 40 to 50 per cent reduction in length 7 to 10 days were required when the tests were conducted at room temperature, 25° ± 2° However, only 3 to 4 days were required when the assay was conducted in an incubator at 30 0° ± 0 5°

A typical dose-response curve for thyroxine is given in Fig 1 Considerable variation in response to thyroxine was observed in experiments carried out at different times, owing to fluctuations in temperature and to differences in the ages of the tadpoles

Goiter Prevention Method—The goiter prevention method for the biological determination of DL-thyroxine or thyroxine-like activity in rats has

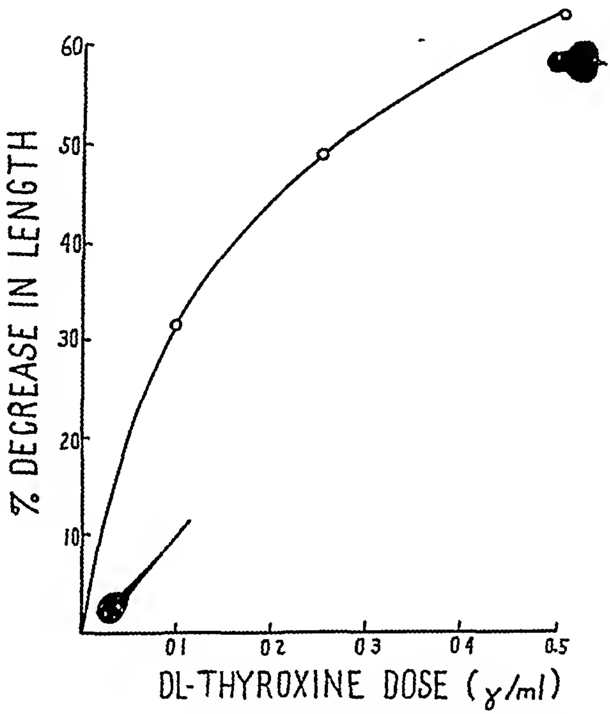


FIG 1 DL-Thyroxine dose plotted against response in terms of per cent decrease in length in a typical experiment with larvae of *Bufo* sp Groups of five tadpoles, 20 to 25 mm in length, were incubated at 25° ± 3° for 7 days or at 30 0° ± 0 5° for 3 to 4 days Initial and final lengths were determined to the nearest 0 5 mm Contrast in body shape of the larvae with increasing DL-thyroxine concentration has also been indicated

been employed by Dempsey and Astwood (15), Reineke, Mixner, and Turner (16), and others Groups of four to six albino rats of the University of Southern California strain, weighing between 100 and 200 gm, were used throughout these experiments Over the 15 day assay period, the experimental animals were fed *ad libitum* with 0 3 per cent thiouracil incorporated in their stock diet Daily intraperitoneal injections of the test compounds were given for 14 days, the injection volume usually being

about 0 25 ml per 100 gm The animals were weighed and sacrificed on the 15th day The thyroid glands were then removed and weighed to the nearest 0 1 mg The room temperature was in the range of 24° ± 1 6° DL-Thyroxine controls were used in each series of experiments A typical

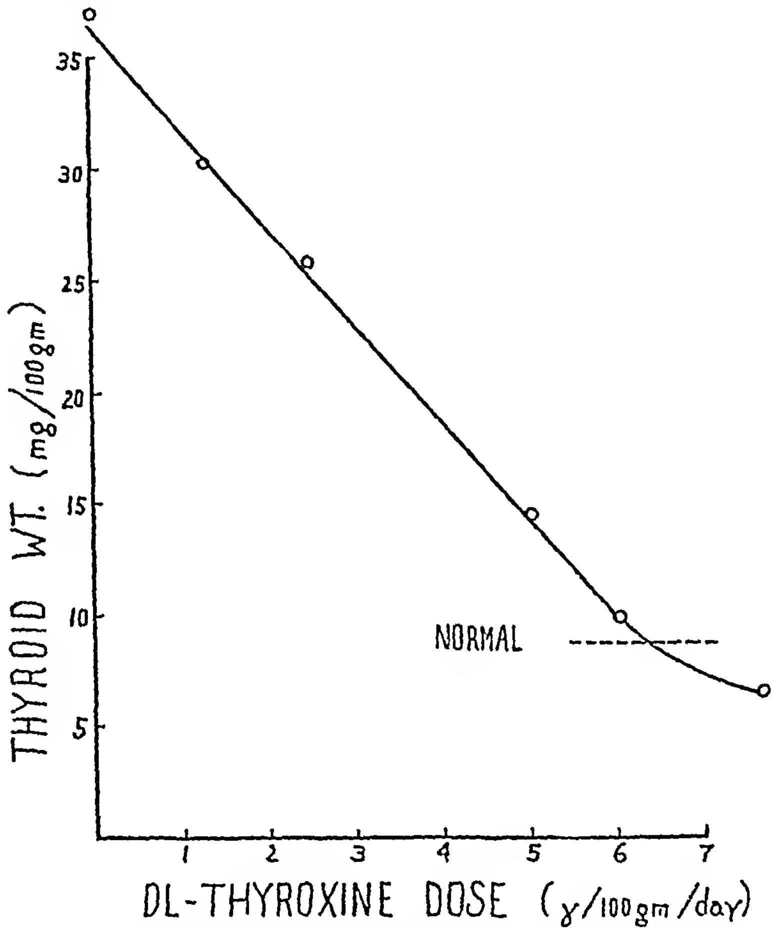


FIG 2 Typical standard curve showing the effect of DL-thyroxine dose, in micrograms per 100 gm of body weight, on the thyroid weight, in mg per 100 gm, in thiouracil-fed rats Groups of approximately six 150 to 200 gm females were fed a 0 3 per cent thiouracil diet for 15 days, during which time they received daily intraperitoneal injections of the appropriate amount of DL-thyroxine

standard curve showing the direct relationship between DL-thyroxine dose and thyroid weight response is shown in Fig 2

RESULTS AND DISCUSSION

The results of the tests on the various compounds are summarized in Tables I and II, which show that 3,5-diiodo-L-tyrosine, the benzoic acid analogue, N-acetylthyroxine, and the glycine homologue of thyroxine exert thyroxine-like activity in both species

It is evident from Table I that under the conditions of this experiment sodium iodide, even at 1 25 mg per ml, had no influence on the body length of tadpoles In longer term experiments, however, it has frequently been observed that iodide ion will accelerate the rate of metamorphosis of amphibian larvae Concentrations of organic iodine containing compounds such as tetraiodophthalate and N-acetyldiiodotyrosine as high as 0 2 and 1 0 mg per ml respectively had no detectable influence on body length

In Table I, diiodotyrosine is shown to exert a pronounced effect on the body length of tadpoles at levels of a few micrograms per ml Thus, the thyroxine-like activity of diiodotyrosine is at least several hundred times greater in these experiments than an equivalent amount of inorganic iodine The thyroxine-like action of 3,5-diiodo-L-tyrosine on the body length and shape of tadpoles ranged from 1 to 2 per cent of that of DL-thyroxine, since 25 γ per ml of diiodotyrosine gave an effect equal to that of about 0 25 γ per ml of thyroxine It cannot yet be stated whether or not this activity is due to the conversion of diiodotyrosine to thyroxine in the incubation medium or in the organism We have observed, however, that diiodotyrosine solutions kept at room temperature for some months show a steadily increasing thyroxine-like action, rising to as high as 6 per cent of that of thyroxine after 12 months of storage

Table I also shows that the thyroxine-like effects of the glycine homologue and benzoic acid analogue of thyroxine on body length and shape of tadpoles are very high Although the possibility that these compounds may be converted to thyroxine cannot be eliminated, we believe it more likely that the thyroxine-like action is inherent in the compounds themselves

It is interesting that acetylation of the amino group of either thyroxine or diiodotyrosine markedly reduces their thyroxine-like action in Amphibia Similar results have been previously reported for N-acetylthyroxine (Kendall (17), Swingle *et al* (18)) This suggests that the acetylated compounds are either used inefficiently as such or are only poorly hydrolyzed to the free amino acids

Table II shows that diiodotyrosine, the glycine homologue, and the benzoic acid analogue show definite thyroxine-like activity in the prevention of thiouracil goiters This action is very definite, although relatively large amounts of the compounds are necessary to prevent thyroid enlargement completely The prevention of thiouracil goiters implies a participation of these compounds directly or indirectly in the thyroid-pituitary axis, and need not necessarily indicate a thyroxine-like influence on peripheral tissue Preliminary experiments on the effect of diiodotyrosine, N-acetylthyroxine, and the benzoic acid analogue on the basal metabolic rates of rats have indicated, however, that a metabolic stimulation also occurs with the administration of these compounds

It is well known that iodide administration partially reduces the goitrogenic action of thiouracil in rats (19) However, in our experience, one

TABLE I

Thyroxine-Like Activity of Compounds As Determined by Amphibian Metamorphosis

Compound	Dose	Per cent decrease in length	Per cent thyroxine like activity*	Average thyroxine like activity of compound
	γ per ml			*per cent*
3,5-Diiodo-L-tyrosine (fresh)	5	9	0.5	
	15	25	2.0	
	25	45	1.0	
	50	64	1.0	
	25	33	1.0	
	50	53	1.0	1.0
Benzoic acid analogue	2.0	18	10	
	3.0	40	15	
	1.5	23	10	
	2.5	41	20	
	4.5	50	12	
	0.5	14	20	
	3.0	31	10	
	1.5	39	30	
Glycine homologue	4.5	65	20	16
	2.5	33	20	
	0.25	25	50	
	1.0	55	50	
	1.5	60	50	
	2.5	37	20	35
N-Acetyl-DL-thyroxine	5.0	10	2	
	12.5	37	3	
	12.5	42	3	
	25.0	55	2	2.5
N-Acetyl-3,5-diiodo-L-tyrosine	500	0	0	
	1000	0	0	
	2500	22	0.005	0.005
Potassium iodide	25	−6	0	
	125	(Toxic)		0
Sodium iodide	50	0	0	
	125	0	0	
	250	0	0	
	500	0	0	
	1250	0	0	0

* Estimated from the DL-thyroxine curve based on untreated controls and several DL-thyroxine levels observed simultaneously with the test substance

experiment being shown in Table II, this effect has never exceeded 35 per cent restoration even at very high iodide levels From the fact that the

effect of thiouracil on the weights of the thyroid gland can be completely negated by the administration of diiodotyrosine or the benzoic acid analogue, it is inferred that these compounds affect the thyroid-pituitary axis independently from the possible liberation of iodide Similarly the significant effect of the glycine homologue at a level of 5 mg per kilo is interpreted as a thyroxine-like action of this compound

TABLE II

Thyroxine-Like Activity of Compounds As Determined by Goiter Prevention Method

Daily intraperitoneal injection	Daily dose	Thyroid weight	Decrease in thiouracil effect*	DL-Thyroxine equivalent†	Per cent DL-thyroxine activity
	mg per 100 gm	*mg per 100 gm*	*per cent*	γ	
3,5-Diiodo-L-tyrosine	2 5	10 6 ± 1 4‡	94	2 5	0 10
	15 0	17 8 ± 3 2	65	4 2	0 028
	25 0	10 8 ± 1 3	95	5 7	0 023
Benzoic acid analogue	0 25	30 4 ± 2 5	20	0 7	0 28
	1 0	23 8 ± 9 9	16	1 1	0 11
	1 0	18 4 ± 1 8	52	1 5	0 15
	5 0	7 3 ± 0 6	112	4 0	0 08
Glycine homologue§	0 50	20 9 ± 3 9	45	1 0	0 20
N-Acetyl-DL-thyroxine	0 0050	24 3 ± 5 1	43	1 5	30
	0 0125	13 1 ± 1 9	72	1 9	15
N-Acetyl-3,5 diiodo-L-tyrosine	5 0	20 6 ± 2 1	57	2 1	0 04
Potassium iodide	25 0	28 1 ± 3 9	32	1 9	<0 008

* Per cent decrease in thiouracil effect = 100 × $(X - Y)/(X - N)$, where X is the thyroid weight of thiouracil controls, Y that observed with the test compound, and N that of normal controls (9 5 mg)

† Estimated from the standard DL thyroxine curve based on the thiouracil controls and one or more DL-thyroxine levels run simultaneously with the substance under test

‡ Average deviation

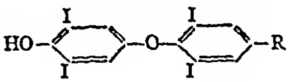
§ Mice were used as test animals for this experiment to permit testing of this compound at a sufficiently high level

N-Acetylthyroxine showed one-sixth to one-third the activity of thyroxine when tested by the goiter prevention method These data are in good agreement with those of Gaddum (6) The relatively greater activity of this compound and of N-acetyldiiodotyrosine in the rat compared to the tadpole is the only case that we have encountered in which compounds exert greater thyroxine-like activity in the higher species The indication of a species difference in the utilization of N-acetylated thyroxine-like compounds may help to explain some of the earlier discrepancies on this point in the literature (6, 17, 18)

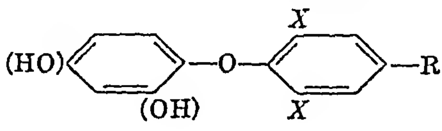
These findings permit some generalizations as to the importance of the side chain in the structural requirements for thyroxine-like activity While the full side chain appears to be necessary for the maximum quantitative effect of a compound, this part of the molecule can nevertheless undergo considerable alteration without eliminating thyroxine-like activity The data available on a number of compounds, involving side chain modifications, are presented in Table III The series is not yet complete, but all compounds involving side chain alterations studied thus far have shown an appreciable thyroxine-like activity on Amphibia and a demonstrable activity on mammals From this work and that of Niemann *et al* (7, 8) we can generalize still further on the structural requirements for thyroxine-like activity The major structural prerequisites, represented by

TABLE III

Effect of Side Chain Variation on Thyroxine-Like Activity

R of 	Per cent thyroxine like activity		
	Amphibia	Mammalia	Bibliographic reference No
COOH	16	0 16	This report
Ethylamine	50–100	50	(3)
Glycine	35	0 20	This report
Pyruvic acid		27	(22)
Alanine	100	100	
" N-acetyl	2 5	10–30	(6), this report
" N-glycyl		100	(6)
" N-alanyl		100	(6)

(II), are as follows [2, 3] (1) an orthodihalogenophenolic-diphenyl ether con-



(II)

figuration, (2) a hydroxy group either ortho or para to the ether oxygen, (3) a side chain which must include a functional group such as a carboxyl or an amino group or both Maximum thyroxine-like activity is obtained

[2] The activity of 3,5-diiodo-L-tyrosine is the only important exception to II This can be explained by assuming *in vivo* conversion to thyroxine This is not unlikely in view of the work of von Mutzenbecher (20) and Dvoskin (21)

[3] Preliminary tests on the effects of 3,5-diiodo-4-(4′-hydroxyphenoxy)-aniline on Amphibia indicate that this compound is at least one-fourth as active as DL-thyroxine, lending further support to the suggestions made Preparation of the corresponding tetraiodo aniline derivative is in progress

when the side chain consists of an alanine group, the hydroxyl group is para to the ether linkage, and there are 4 iodine atoms occupying the 3,5 and 3′,5′ positions

SUMMARY

Several compounds structurally related to thyroxine have been tested for thyroxine-like activity on amphibian metamorphosis and prevention of increase in the thyroid gland weights of thiouracil-fed rats Demonstrable activity has been observed for the glycine homologue of thyroxine, a benzoic acid analogue of thyroxine, N-acetylthyroxine, and 3,5-diiodo-L-tyrosine in both tests These findings indicate a lack of specificity of the side chain requirement for thyroxine-like activity

It is suggested that the minimum structural requirements for thyroxine-like activity in Amphibia and mammals are the orthodihalogenophenolic-diphenyl ether configuration, a hydroxy group ortho or para to the ether oxygen, and a side chain which includes some functional group

We are indebted to Miss Elizabeth V Tukich for assistance in the goiter prevention assays

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A FACTOR REQUIRED FOR THE GROWTH OF LEUCONOSTOC CITROVORUM*

By H E SAUBERLICH and C A BAUMANN

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During a survey of organisms suitable for the microbiological determination of alanine, it was found that *Leuconostoc citrovorum* 8081 failed to grow on a synthetic medium that was satisfactory for *Leuconostoc mesenteroides* P-60 and other assay organisms However, the addition to the medium of certain crude extracts from liver resulted in a very rapid rate of growth of *L citrovorum*, and the commercial liver preparation reticulogen (Lilly), active in Addison's pernicious anemia, proved to be effective in such small concentrations that the organism could be used for the microbiological determination of alanine and other amino acids in the presence of this supplement Preliminary attempts were then made to determine the relationship between the unknown factor required by *L citrovorum* and certain other growth factors, including the antipernicious anemia principle

Methods

The organism, *Leuconostoc citrovorum* 8081, American Type Culture Collection, Georgetown University, Washington, D C, was maintained on a yeast-peptone stab medium and was transferred every 2 to 4 weeks Prior to use for assay purposes, the organism was grown in 10 ml of a casein hydrolysate (Medium IV) (1) to which 0 2 per cent of yeast extract (Difco) had been added A 20 to 24 hour culture was centrifuged twice and the cells diluted with 0 9 per cent NaCl to a standard degree of turbidity (G = 65 to 70 in a standard Evelyn tube, Filter 660) 1 drop of this diluted cell suspension was added to each assay tube, which also contained 1 ml of the synthetic medium (Table I) plus the sample and water to make a total of 2 ml The assay tubes were then incubated for 72 hours at 37° and the acid production measured by electrometric titration with 0 02 N NaOH

EXPERIMENTAL

Response to Liver Preparations—The addition of the liver concentrate reticulogen, which according to the manufacturer contained 20 U S P

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units of the antipernicious anemia principle per ml , resulted in such rapid growth of the organism *Leuconostoc citrovorum* that a quantitative response could be measured turbidimetrically after only 10 hours (Fig 1) A maximal response was observed when the medium contained 2 0 microliters of reticulogen per 10 ml of medium as read, or 0 2 microliter per ml However, when the incubation was prolonged for 72 hours, maximal acid production was observed with only 0 09 to 0 10 microliter of reticulogen per ml of medium as titrated (0 2 microliter per 2 ml tube, Fig 2), and a half optimal response was observed when the medium contained only 0 025 microliter of reticulogen per ml This latter half optimal response was designated as 1 "*citrovorum* unit" for the assay of other crude materials to obviate the necessity for a specific reference standard The response of *L citrovorum* to the active principle in liver was not altered by the addition

TABLE I

*Basal Medium VI**

	mg		mg
Amino acids		Salts A	
DL-α-Alanine	200	KH_2PO_4	600
L-Arginine	200	K_2HPO_4	600
L-Aspartic acid	100	Salts B	
L-Asparagine	400	$MgSO_4 \cdot 7H_2O$	200
L-Cysteine	50	$FeSO_4 \cdot 7H_2O$	10
L-Glutamic acid	300	$MnSO_4 \cdot 4H_2O$	20
Glycine	100	NaCl	10
L-Histidine	50	Purines and pyrimidines	
DL-Isoleucine	250	Adenine sulfate $2H_2O$	10
DL-Leucine	250	Guanine hydrochloride	10
L-Lysine	200	Uracil	10
DL-Methionine	100	Xanthine	10
DL-Phenylalanine	100	Vitamins	
L-Proline	100	Thiamine hydrochloride	0 5
DL-Serine	50	Pyridoxine hydrochloride	1 0
DL-Threonine	200	Pyridoxamine hydrochloride	0 3
DL-Tryptophan	40	Pyridoxal hydrochloride	0 3
L-Tyrosine	100	Calcium *dl*-pantothenate	0 5
DL-Valine	250	Riboflavin	0 5
	gm	Nicotinic acid	1 0
Glucose	25	*p*-Aminobenzoic acid	0 1
Sodium acetate	20	Biotin	0 001
Ammonium chloride	3	Folic acid	0 010
		Adjust to pH 6 8, add distilled H_2O to	500 ml

* The designation, Medium VI, is used to distinguish this medium from those described in previous publications (1)

of large amounts of thiamine, pyridoxal, pyridoxamine, hydroxyproline, or the ash of yeast extract Some production of acid, however, resulted when large amounts of crystalline folic acid were present in the medium A response equivalent to 1 *citrovorum* unit was noted when 1 4 to 2 γ of

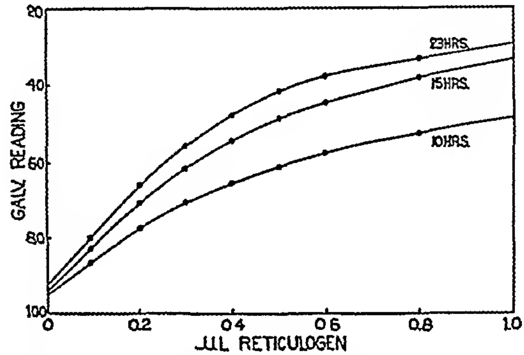

FIG 1 Response of *Leuconostoc citrovorum* to graded amounts of reticulogen as determined by turbidity measurements after 10, 15, and 23 hours with the Evelyn colorimeter (660 mμ filter with an uninoculated tube set at 100 as the blank, 10 ml volume assay)

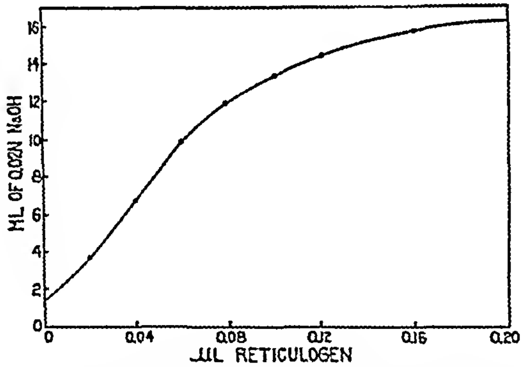

FIG 2 Response of *Leuconostoc citrovorum* to graded amounts of reticulogen, as determined by the measurement of acid production after 72 hours (2 ml volume assay)

folic acid were added per ml of medium as titrated The response of the organism to folic acid, however, differed qualitatively from that due to the factor in liver, since there was a marked delay in the growth of the organism given only the folic acid, and no visible growth was noted during the first 24 to 36 hours Since the reticulogen preparation contained only 8 7 γ of folic acid per ml, the amount of folic acid in the 0 025 microliter of reticu-

logen required for half optimal growth supplied only 0 0002 γ of folic acid per ml of medium Thus it was obvious that the response of the organism to reticulogen could not be attributed to the folic acid present, for the amount of folic acid in the basal medium was 0 02 γ per ml The data by Dunn and associates indicated that *L citrovorum* 8081 grew fairly well on

TABLE II

Response of Leuconostoc citrovorum 8081 to Various Liver Concentrates Active against Pernicious Anemia

Sample No	Liver extract*	U S P units per ml	*Citrovorum* units† per ml
1	Lilly, Lot 360, reticulogen, enzyme-treated	20	41,900
2	" " 360, " untreated	20	40,000
3	" " 360 "	20	35,000
4	Sharp and Dohme, Lot 2505	15	34,000
5	Abbott, Lot E-1043	15	32,000
6	Lilly, Lot 370	2	20,200
7	Abbott, Lot E-1125	15	19,200
8	Lilly, Lot E-1181	Unknown	18,100
9	" " E-1203, per gm	"	13,900
10	" " E-1204	"	11,720
11	" " 410	10	10,600
12	" " E-1180	Unknown	9,180
13	Abbott, Lot E-567	15	7,880
14	Lilly, Lot E-1108	20	6,710
15	Armour	10	4,930
16	Lederle, Lot 3641-34	10	4,880
17	Abbott, 95% alcohol extractives, per gm	Unknown	2,140

* Sample 1, Lot 0030-442346, was treated with hog kidney enzyme in the manner used to liberate conjugated folic acid (4), Sample 3 was an unhydrolyzed sample of similar material, Lot 4250-418561, Samples 3 to 17 were obtained from Dr W R Ruegamer, U D Register, Dr C A Elvehjem, Dr A R Robblee, C A Nichol, Dr W W Cravens, J J Betheil, and Dr H A Lardy of this department, Sample 6 was a relatively crude preparation high in solids, Sample 14 was a relatively purified preparation low in solids

† The "*citrovorum* unit" was designated as the amount of substance required per ml to give one-half maximal acid production in 72 hours by *Leuconostoc citrovorum* 8081 when grown on Basal Medium VI (Table I)

a medium containing 0 005 γ per ml of a crude concentrate of folic acid (2), but that the organism failed to grow when the concentrate was replaced by crystalline folic acid (3)

A series of preparations from liver ranged in activity from 2140 to 41,900 *citrovorum* units per ml or gm (Table II) Most of these were commercial products or experimental samples prepared as concentrates of the anti-pernicious anemia principle All of the preparations which were active

clinically were also active in promoting the growth of *Leuconostoc citrovorum* Quantitatively, however, there was a fairly wide discrepancy between the potencies of the various preparations for the growth of *Leuconostoc citrovorum* and their reputed effectiveness for pernicious anemia patients The most active preparation for the organism was a sample of the liver concentrate, reticulogen, which had been treated with hog kidney enzymes for 24 hours at pH 4 5 in a citrate-phosphate buffer This treatment is effective in the release of folic acid from its conjugate (4) This preparation contained 41,900 *citrovorum* units per ml (Table II, Sample 1), whereas an unhydrolyzed aliquot of the same preparation, reticulogen (Table II, Sample 2), contained 40,000 *citrovorum* units per ml The hydrolytic procedure therefore failed to increase the activity of the preparation by more than 5 per cent Since Sample 2 contained 20 U S P (clinical) units of the antipernicious anemia factor and 40,000 *citrovorum* units per ml, 1 U S P unit of this preparation contained 2000 *citrovorum* units Essentially the same relationship was observed in a second reticulogen preparation, Sample 3, and in liver extracts Samples 4 and 5, in which 15 U S P units per ml were equivalent to 34,000 and 32,000 *citrovorum* units respectively (Table II) However, two other preparations from liver containing 15 U S P units per ml (Samples 13 and 7) were found to contain only 7880 and 19,200 *citrovorum* units per ml The most active preparation for the organism in relation to reputed clinical potency was a crude preparation relatively high in solids in which 2 U S P units corresponded to 20,200 *citrovorum* units (Table II, Sample 6), a ratio of 1 10,100 On the other hand, a highly purified preparation low in solids, Sample 14, which was reputed to contain 20 U S P units per ml, was found to contain only 6700 *citrovorum* units per ml, a ratio of only 1 335 The ratio of clinical to microbiological response for all the samples was thus found to vary over a 30-fold range This result is suggestive of the experience of Rickes and associates (5) with *Lactobacillus lactis* Dorner, in which the ratios of clinical to microbiological potencies of products containing 15 U S P units per ml varied over a 12-fold range

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obtained with turbidimetric measurements after 10 to 15 hours In the assay of folic acid for *citrovorum* units, however, turbidimetric assays yielded a value of zero, due to the delay in the growth response

TABLE III

Growth of Leuconostoc citrovorum 8081 on Medium VI Fortified with Various Substances

Sample No	Substance added*	*Citrovorum* units
18	Fish solubles (80% ethyl alcohol extract) per ml	179
19	" " (BuOH-soluble) per ml	137
20	" " (" pH 3) per ml	132
21	" " (BuOH-insoluble) per ml	24
22	" " (") " "	29
23	" " (BuOH-soluble, pH 10) per ml	16
24	" " (" " 7) " "	16
25	Yeast extract (Difco) per gm	4720
26	Bacto-peptone " " "	3240
27	Rice-bran concentrate (Vitab) per gm	625
28	Folic acid (at 1 γ per ml medium) per mg	480
	" " (" 2" " " ") " "	1540
29	Formyl folic acid (at 4 γ per ml medium) per mg	470
30	Milk (pasteurized) per ml	Very slight
31	Thymine (at 10-300 γ per ml medium)	Inactive
32	Xanthopterin (at 2-60 γ per ml medium)	"
33	Leucopterin (" 2-60 " " " ")	"
34	Yeast extract	"
35	Thymine desoxyriboside (at 0 4 γ per ml medium) per mg	2720

* Samples 18 to 24 were obtained from Dr A R Robblee, C A Nichol, Dr W W Cravens, and Dr C A Elvehjem of this department The preparation of these samples and their activities in chickens have been published (6, 7), or described in a thesis entitled "Studies on unidentified chick growth factors," Dr A R Robblee, University of Wisconsin (1948) Sample 29 was obtained from J J Betheil and Dr H A Lardy, Sample 31 from H P Broquist and Dr E E Snell, Sample 32 from B H Olson and Dr W H Peterson, Sample 33 from Dr A R Robblee, C A Nichol, Dr W W Cravens, and Dr C A Elvehjem, Sample 34 (S) from W L Williams and Dr E E Snell, and Sample 35 from Dr E E Snell

Chick Factors—Robblee and associates (6, 7) have found that a factor in certain crude materials is needed for the optimal growth of chicks fed all of the known vitamins available in pure crystalline form The active principle is present in concentrates of fish solubles and in certain liver preparations, including reticulogen Assays of various preparations from fish solubles, obtained from Messrs Robblee, Nichol, and Elvehjem of this department, indicated that most of the preparations were only slightly or moderately active for *Leuconostoc citrovorum* (Table III) A preparation

insoluble in butyl alcohol, Sample 21, which was very active in the chick,[1] contained only 24 *citrovorum* units per ml , on the other hand, Sample 19, a fraction soluble in butyl alcohol, contained 137 units per ml for the organism, but was inactive for the chicken [1] Thus it would appear that the factors responsible for the respective responses in the two species are not identical This is also suggested by the quantitative responses of the two species to the liver extracts Samples 5 and 13, respectively, the former was inactive in the chick assay,[1] although it proved to be one of the most potent concentrates of the *citrovorum* factor thus far encountered On the other hand, Sample 13, which was very active in the chicken,[1] was only one-fourth as active as Sample 5 for the microorganism

The factor required by *Leuconostoc citrovorum* appears to be different from the unknown principle required by a special strain of *Lactobacillus bulgaricus* (8) A preparation from yeast[2] that was very active for the latter organism[3] proved to be inactive for *L citrovorum* Furthermore, the factor required by *L citrovorum* did not appear to coincide with the principle required for the optimal growth of hyperthyroid rats (9) as indicated in preliminary assays Sample 13, which was highly active in the rat,[4] contained only 7880 *citrovorum* units per ml as contrasted to Preparation 5, which was relatively inactive in the rat[4] but contained 32,000 *citrovorum* units per ml (Table II)

Crude materials which stimulated the growth of *Leuconostoc citrovorum* included yeast extract, Bacto-peptone, and a rice-bran concentrate (Vitab), while milk contained very slight activity Formyl folic acid[5] proved somewhat less active than folic acid itself, whereas xanthopterin, leucopterin, and thymine, when added at levels of 2 to 60 γ per ml , 2 to 60 γ per ml , and 10 to 300 γ per ml , respectively, were inactive for *Leuconostoc citrovorum*

A sample of thymine desoxyriboside[2] stimulated the initial growth of *Leuconostoc citrovorum* 8081 in concentrations up to 0 6 γ per ml , but a further increase in the concentration of the riboside up to 2 0 γ per ml failed to increase turbidity further The maximal turbidity reached in 16 hours by the cultures containing the riboside was less than one-fourth that reached by cultures containing reticulogen An intermediate concentration of the thymidine, 0 1 γ per ml , stimulated growth equivalent to that observed when 0 01 microliter of reticulogen was present per ml If the latter re-

[1] Personal communication with A R Robblee, C A Nichol, W W Cravens, and C A Elvehjem

[2] We are indebted to W L Williams and E E Snell for this preparation

[3] Personal communication with W L Williams and E E Snell

[4] Personal communication with U D Register W R Ruegamer, and C A Elvehjem

[5] Obtained from J J Betheil and H A Lardy

sponse were due only to thymidine, this would indicate a thymidine content of 10 per cent in the solids of reticulogen

A similar result was obtained when the relative activities were based on the production of acid after 72 hours But whereas increasing concentrations of reticulogen resulted in marked increases in the amounts of acid produced, further increases in the concentration of thymidine stimulated further acid production only slightly, with the result that the amounts of acid produced by cultures containing 1 0 γ of thymidine per ml corresponded to an apparent thymidine concentration of 23 per cent in the solids of the liver concentrate Thus, since the response of *Leuconostoc citrovorum* 8081 to thymidine differed qualitatively from that to the liver concentrate, it was concluded that some other active agent or agents must have been present in the latter preparation

DISCUSSION

The fact that a 30-fold variation was encountered between U S P units of the antipernicious anemia factor and the *citrovorum* units found in various preparations of liver might suggest that there is no relationship whatever between the antipernicious anemia principle and the factor or factors stimulating the growth of *Leuconostoc citrovorum* However, when *Lactobacillus lactis* Dorner was used as the assay organism for products containing 15 U S P units of the antipernicious anemia factor per ml, the ratios of clinical to microbiological units varied over a 12-fold range (5) Nevertheless, this organism was used successfully as an aid in the isolation of vitamin B_{12} Analyses with *L citrovorum* of Samples 4, 5, 7, and 13 (Table II), preparations containing 15 U S P units per ml, indicated that the ratio of clinical to *citrovorum* units varied over only about a 4-fold range Thus it is possible that *L citrovorum* may prove to be superior to *Lactobacillus lactis* Dorner as an assay organism for the antipernicious anemia factor, although this cannot be determined until the details for the use of the latter organism are revealed In any event, an exact correlation between the results of microbiological methods and those of clinical assays should not be expected, in view of the variations inherent in clinical responses

Another reason for suspecting a metabolic connection between *Leuconostoc citrovorum* and pernicious anemia patients is the peculiar partial response of both to high levels of folic acid High levels of this vitamin produce a partial remission of the symptoms of the disease without, however, curing all the symptoms benefited by crude concentrates from liver (10, 11) Similarly, high amounts of folic acid stimulate the growth of *Leuconostoc citrovorum* but in a delayed manner, qualitatively different from that observed with the best preparations from liver One might, therefore,

postulate a similarity in structure between folic acid and the natural agents active in the organism and the pernicious anemia patient Another possibility is that folic acid is necessary for the synthesis of the unknown principles

SUMMARY

1 *Leuconostoc citrovorum* 8081 failed to grow on a synthetic medium adequate for the growth of *Leuconostoc mesenteroides* P-60 and other common assay organisms Growth was rapid in the presence of certain concentrates from liver, peptone, or yeast extract

2 All liver preparations known to be active against pernicious anemia were also active for *Leuconostoc citrovorum*, but a 30-fold variation was found between preparations in the ratios of microbiological activity to reputed clinical potency

3 The factor required by *Leuconostoc citrovorum* did not appear to be identical with the factor in fish solubles required by the chick, with the factor in liver required for the optimal growth of hyperthyroid rats, nor with the factor in yeast required by a strain of *Lactobacillus bulgaricus*

4 High amounts of folic acid stimulated maximal acid production by *Leuconostoc citrovorum*, provided the culture was incubated for 72 hours Qualitatively, however, the growth of the organism was much slower in the presence of high amounts of folic acid than when small amounts of liver preparations were present in the medium

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CORRECTIONS OF PUBLISHED ELECTROPHORETIC MOBILITIES OF ADRENOCORTICOTROPIC AND PARATHYROID HORMONES

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A recent check-up of the electrophoresis apparatus in use at the Laboratory of Physiological Chemistry at Yale has revealed that the milliammeter of the power supply is shunted, so that the actual current delivered by the power supply is 3 4 times the current indicated on the meter Since the direct milliammeter readings were used in the calculations of electrophoretic mobilities of the adrenocorticotropic hormone, published by Sayers, White, and Long (1), and of the components of the parathyroid hormone preparation described by L'Heureux, Tepperman, and Wilhelmi (2), the values obtained were all too high by a factor of 3 4 The corrected values are briefly summarized as follows

For adrenocorticotropic hormone (1)

Acetate buffer,	ionic	strength	0 1,	pH 3 26 =	$+7\ 1 \times 10^{-5}$	cm ^{2}	volt^{-1}	sec $^{-1}$
"	"	"	0 13,	" 4 13 =	$+2\ 5 \times 10^{-5}$	"	"	"
Phosphate "	"	"	0 2,	" 6 37 =	$-7\ 1 \times 10^{-5}$	"	"	"
"	"	"	0 2,	" 7 95 =	$-8\ 3 \times 10^{-5}$	"	"	"

For parathyroid hormone (2), in acetate buffer, ionic strength 0 1, pH 3 50, fast component = $+8\ 8 \times 10^{-5}$ cm ^{2} volt^{-1} sec^{-1}, slow component = $+6\ 8 \times 10^{-5}$ cm^{2} volt^{-1} sec^{-1}

If one makes allowance for the differences in ionic strengths of the buffers used by the two groups of investigators, the corrected values for the electrophoretic mobilites of adrenocorticotropic hormone reported by Sayers *et al* are now in substantial agreement with those reported by Li, Evans, and Simpson (3)

The authors apologize for their lack of complete understanding of their instrument They hope that the foregoing corrections may help to avoid misunderstanding and confusion

SUMMARY

The values for the electrophoretic mobilities of the adrenocorticotropic hormone published by Sayers *et al* and for those of a parathyroid hormone preparation published by L'Heureux *et al* have been found to be too large

by a factor of 3 4 When this correction is applied to the mobilities of the adrenocorticotropic hormone, the new values are seen to be in substantial agreement with those reported for this hormone by Li, Evans, and Simpson

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PREPARATION AND SOME PROPERTIES OF HYALURONIC ACID FROM UMBILICAL CORD OF THE PIG*

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Within the last 13 years hyaluronic acid has been isolated from a variety of sources (1) umbilical cord, vitreous humor, synovial fluid, tumors, and hemolytic streptococci These mucopolysaccharides all have essentially the same chemical composition, equimolecular portions of acetylglucosamine and glucuronic acid, but vary greatly in viscosity Hyaluronic acid was first isolated by Meyer and Palmer (2) in 1934 from bovine vitreous humor Human umbilical cord was shown by them (3) to be a rich source of hyaluronic acid Recently Hadidian and Pirie (4) have prepared from this same source a product more viscous than any hitherto obtained However, the unbilical cord of no species other than the human has yet been investigated as a source of hyaluronic acid It was the purpose of this work to investigate pig umbilical cord as a source and examine the properties of the hyaluronic acid derived

EXPERIMENTAL

Fractionation—Fresh frozen pig umbilical cords were used as the starting material in this work In previous work, human umbilical cords were stored in acetone 1 to 6 weeks before processing In this work, the effect of this acetone storage on the extraction procedures was studied by extracting a portion of fresh frozen pig umbilical cords as received, while a second portion was set aside in acetone for 6 weeks before the extraction of hyaluronic acid The method of extracting hyaluronic acid was essentially the fractionation technique described by Hadidian and Pirie (4) The umbilical cords were cut up and extracted with water and the residue was ground and extracted with 0 1 M sodium chloride At this point in the procedure an innovation was introduced, the residue from the sodium chloride treatment was extracted with boiling water The residue was suspended in 0 01 M hydrochloric acid (the pH readjusted to 2 when necessary) and

* This investigation was aided by a grant from the L Farber Company of Worcester, Massachusetts Pig umbilical cords were obtained through the courtesy of G D Searle and Company, Chicago, Illinois

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digested at 37° with pepsin, followed by trypsin at pH 7 6 Hyaluronic acid was precipitated from the respective fluids in the following manner Hydrochloric acid was added and formed a "mucin clot" which was removed, $1\frac{1}{3}$ volumes of ethanol were added to the acid fluid and the precipitate removed, solid ammonium sulfate was added to the alcoholic supernatant fluid with vigorous mechanical agitation, and on standing the system separated into two layers with hyaluronic acid appearing at the interface A precipitate was not always obtained at each step in the procedure

In this manner, both the fresh frozen pig umbilical cords and the acetone-stored cords were extracted For purposes of comparison, human umbilical cords (acetone-stored) were extracted according to the above procedure Supplementary work was done with pig cords by grinding fresh frozen material, and the entire mass incubated with pepsin at 37°, followed by trypsin This digested fluid was put through the acid-alcohol-ammonium sulfate fractionation

Purification—The mucin clots were partially fractionated by precipitation with alcoholic potassium acetate from alkaline solution, as in McClean's (5) procedure, or incubation with pepsin and trypsin, the digestion procedure being more satisfactory usually

Several of the preparations referred to in Table I were made by removing the protein by shaking with a mixture of 1 part chloroform and 2 parts amyl alcohol (volume per volume) according to the Sevag technique (6) The hyaluronic acid was precipitated from acid solution by $1\frac{1}{3}$ volumes of ethanol, then suspended in a smaller volume of water and dialyzed

Chemical Analyses—The nitrogen content was determined by the micro-Kjeldahl procedure The acetyl content was measured by hydrolyzing a 1 ml sample in 2 5 N H_2SO_4 at 100° for 75 minutes, steam-distilling in the apparatus described by Markham (7), and titrating the distillate with N/75 NaOH The glucosamine determinations were made by the method of Elson and Morgan (8) on material that had been hydrolyzed for 6 to 8 hours in 5 N HCl at 100° and then evaporated to dryness in a vacuum desiccator over a moistened caustic All of these analyses were made on aliquots of dialyzed solutions whose concentration had been determined by drying at 100° for 2 hours and cooled in a desiccator The values refer to the free acid and not to a salt

Viscosity Measurements—Viscosity measurements were made in an Ostwald viscosimeter with a capillary 9 cm long and having a flow time of about 30 seconds for 4 ml of distilled water This volume was used in all the experiments reported, and all the measurements were made at 25° The standard salt concentrations used throughout were 0 05 M NaCl and 0 05 M phosphate buffer at pH 7 0 This concentration of salt and buffer

TABLE I

Analyses of Mucopolysaccharides Isolated from Umbilical Cord

Extraction	Sample No	Treatment	Nitrogen	Glucosamine	Acetyl	Relative viscosity	Half time
A Pig (fresh frozen)							
			per cent	*per cent*	*per cent*		*sec*
0 1 M NaCl	1	Interfacial	12 1			1 01	
Hot water	2	"	5 8			1 02	
Pepsin trypsin digest	3	Portion, 1 33 vols ethanol	5 5	20 0	6 6	1 04	
" "	4	Bulk, 1 33 vols ethanol	5 3		7 9	1 06	
" "	5	Samples 3 and 4 combined, Sevag purification	3 5	31 4	7 7	1 08	
Mucin clots	6	Alcoholic potassium acetate purification	3 7	23 4	6 6	1 03	
" "	7	Sample 6, Sevag purification	3 1	34 6	7 9	1 03	
Yield gross, 13 2%, corrected to 3 6% N, 5 4%							
B Pig (fresh frozen, acetone stored)							
0 1 M NaCl	10	Mucin clot, alcoholic CH_3-COOK purification	6 5			1 03	
0 1 " "	11	1 33 vols ethanol	5 2	31 5	8 1	1 31	760
0 1 " "	12	Sample 11, Sevag purification	3 2	37 9	10 2	1 38	482
0 1 " "	13	Interfacial	9 5			1 03	
Hot water	14	" digested, 1 33 vols ethanol	3 5	34 9	8 5	1 15	920
Pepsin-trypsin digest	15	Mucin clot	10 6			1 01	
" "	16	1 33 vols ethanol	5 4	34 1	6 5	1 07	
" "	17	Sample 16, Sevag purification	3 2	31 3	9 0	1 08	
Yield gross, 5 3%, corrected to 3 6% N, 3 3%							
C Pig (fresh frozen)							
Pepsin trypsin digest	20	1 33 vols ethanol	5 6	30 6	8 1	1 17	
" "	21	Sample 20, Sevag purification	3 6	29 0	7 8	1 15	957
Yield gross, 10 0%, corrected to 3 6% N, 6 6%							

TABLE I—*Concluded*

Extraction	Sample No	Treatment	Nitrogen	Glucosamine	Acetyl	Relative viscosity	Half time
D Human (acetone-stored)							
			per cent	*per cent*	*per cent*		*sec*
Initial water	30	Interfacial	3 2				
0 1 M NaCl	31	"	3 3	30 8	10 2	1 53	285
0 1 " "	32	Sample 31, Sevag purification	3 0	41 8	11 4	1 49	206
Hot water	33	Interfacial	14 8			1 02	
Initial water	34	1 33 vols ethanol	5 0			1 59	
" "	35	Sample 34, Sevag purification	3 4	43 0	8 6	1 51	293
Initial water, 0 1 M NaCl, hot water combined	36	Mucin clots digested, HCl ppt	2 6	11 2	5 0	1 03	
" "	37	Mucin clots digested, 1 33 vols ethanol	4 0	35 8	7 7	1 23	1244
" "	38	Sample 37, Sevag purification	3 4	34 4	9 2	1 20	735
0 1 M NaCl, hot water combined	39	1 33 vols ethanol	3 1			1 93	
" "	40	Sample 39, Sevag purification	2 8	41 7	11 2	1 87	146

Yield gross, 4 9%, corrected to 3 6% N, 4 4%

was used, since it was the one found by Hadidian and Pirie (9) to give optimum results The term "relative viscosity" refers to the ratio of flow time of the test solution to the flow time of a solution with the same salt concentration For measuring the relative viscosity of hyaluronic acid preparations a standard concentration of 0 3 gm per liter was adopted

The rate at which hyaluronic acid preparations lost viscosity with bull testis hyaluronidase was measured by incubation at 25° with the enzyme in the standard ionic environment The time required for the viscosity to fall half way from the initial to the presumed final value was taken as a measure of the rate of enzyme action Half time measurements are customary in studies of this enzyme Partially purified bull testis enzyme (Schering) was used in all of these experiments at a concentration of 50 γ in 4 ml of test solution

Preparation of Inhibitors

Nitration—A few hyaluronic acid preparations were nitrated to test their inhibition of hyaluronidase hydrolysis of hyaluronic acid The nitration

procedure used was that described by Hadidian and Pirie (9) Both the acid-insoluble and the acid-soluble portions were tested for their inhibitory effect at a concentration of 0 03 gm per liter The nitrogen content was determined by the micro-Kjeldahl method modified to include the nitrogen of nitrates

Acetylation—Preparations from pig cord were acetylated to study their inhibitory effect on the hyaluronidase-hyaluronic acid system The acetylation procedures used were the two described by Hadidian and Pirie (9) In the sulfuric acid method acetylation was allowed to proceed for 30 to 45 minutes at 27° Acetylation was continued for 2 hours at 20° with the pyridine method The acetyl content was determined by the method used above for hyaluronic acid preparations and included both O-acetyl and N-acetyl

Results

Umbilical Cord—Pig umbilical cord proved to be a rich source of hyaluronic acid, nearly as rich as human cord The yield of hyaluronic acid obtained from acetone-stored material was 3 3 per cent of the dry weight of the pig cord as compared with 4 4 per cent for human cord (these values corrected to 3 6 per cent nitrogen)

The most striking difference observed (Table I) for the series of pig cord preparations was in the relative viscosity, all fresh pig cord preparations were very low in viscosity, whereas one product in particular from defatted pig cord (Sample 12) with a relative viscosity of 2 3, calculated to a concentration of 1 gm per liter, compared very favorably with the better preparations from various sources reported by other investigators A compilation of these has been made by Hadidian and Pirie (4) The values for the chemical constituents of preparations from fresh frozen cords were lower than those from defatted cords It was also noted that for pig cord in general the viscous preparations contained 8 5 to 10 2 per cent acetyl, but preparations made by enzymic digestion of residues had lower acetyl contents, as low as 6 5 per cent There was a comparable deficiency in the glucosamine content Viscous products contained 35 to 38 per cent, whereas the non-viscous had less than 35 per cent The nitrogen values were in the range of 3 1 to 3 5 per cent

The fresh frozen pig cords which were ground and subjected to pepsin-trypsin digestion without prior treatment produced the highest yield of hyaluronic acid Since no fractionation was involved in this procedure, the relative viscosity and acetyl and glucosamine values were midway between those for the viscous and non-viscous preparations obtained previously This was, of necessity, anticipated, as this preparation represented the average of the heterogeneous hyaluronic acid fractions present As has

been observed in previous work precipitating hyaluronic acid, the pepsin-trypsin digestion solution yielded no mucin clot with concentrated HCl and no interfacial material separated, but the precipitate was obtained with alcohol This crude material was not excessively contaminated with protein, the nitrogen content was 5 6 per cent which dropped to 3 6 per cent on purification by the Sevag technique followed by dialysis The half time of 957 seconds for Sample 21 was about what would be expected from a preparation having a relative viscosity of 1 15

Inhibitors—Three pig cord products, Samples 5, 12, 21, were nitrated, both the acid-insoluble and the acid-soluble fractions were tested for their inhibiting effect on the hyaluronidase-hyaluronic acid system Sample 5 was non-viscous, Sample 12 the most viscous pig product, and Sample 21

TABLE II

Inhibitors, Nitrated and Acetylated

In these experiments the inhibitor was measured at a final concentration of 0 03 gm per liter The half time was found in the usual manner, and the amount of inhibition given in the right-hand column was derived by dividing the half time found in the presence of the inhibitor by that found in the control experiment with the enzyme and substrate alone

Description of inhibitor	Inhibition
Sample 5 Nitrated, acid-insoluble	2 1
" 12 " "	1 6
Samples 5, 12 Nitrated separately, acid-soluble fractions combined	4 8
Sample 21 Nitrated, acid-insoluble, 5 9% N	1 5
" 21 " acid-soluble, 4 7% N	2 1
" 21 Acetylated, sulfuric acid method, 18 2% acetyl	1 3
" 21 " pyridine method, 18 4% acetyl	1 2

intermediate The acid-soluble fractions of Samples 5 and 12 were combined before measuring their inhibition This was done because of the similarity in the inhibition given by their acid-insoluble products The values obtained for all of the inhibitors with a brief description of the derivation of each are given in Table II The acid-soluble products were found to be better inhibitors than the acid-insoluble fractions Of these nitrated products, the one derived from the non-viscous preparation gave the greatest inhibition Nitrogen analyses (modified micro-Kjeldahl) made on the nitrated products from Sample 21 indicated that the acid-insoluble material was nitrated to the extent of one $—NO_2$ group, and the acid-soluble one-half an $—NO_2$ group The inhibition found for these nitrated pig cord preparations was of about the same order of magnitude as that reported by Hadidian and Pirie (9) for their inhibitors made from human cord products

One hyaluronic acid preparation from pig cord was acetylated by both the sulfuric acid catalyst method and the pyridine method, by each method the resulting product contained about 18 per cent acetyl Acetylated products were found to be poorer inhibitors than the nitrated acid-soluble products

DISCUSSION

Hyaluronic acid can be extracted from pig cord and purified by the same methods that have been used for human umbilical cord The products prepared from pig cord contained practically the same proportion of nitrogen, acetyl, and glucosamine as preparations from human cord The general trend in the physical and chemical properties noted by Hadidian and Pirie (4) for human cord preparations was found here to be the same for pig cord, *i e*, mucin clots had the lowest viscosity with material from pepsin-trypsin-digested extracts intermediate between those and the viscous products, and the non-viscous preparations contained less than the theoretical percentage of both acetyl and glucosamine Preparations of hyaluronic acid from pig cord were hydrolyzed by the enzyme hyaluronidase (testis) Nitrated and acetylated pig preparations were found to inhibit the hyaluronidase-hyaluronic acid system to the same extent as inhibitors made from human cord

In comparing the physical and chemical properties of hyaluronic acid preparations from pig umbilical cord with those from human cord, the most obvious difference was found in the relative viscosity, the human cord products were appreciably more viscous However, the half time of products from both sources was found to be inversely proportional to the relative viscosity The acetyl and glucosamine content of human preparations ranged, in general, higher than in pig preparations These differences in properties observed may be attributable to a species difference

The quality of pig cord preparations extracted was enhanced by acetone storage prior to processing for hyaluronic acid Apparently a protein denaturation process was involved which decreased the solubility of the proteins since the crude products from the fresh frozen pig cords were contaminated with protein to a far greater extent than those from the defatted pig cords The innovation of adding a boiling water treatment to the residue from the 0 1 M NaCl extraction in the procedure was of no particular efficacy A substantial amount of protein was extracted by boiling water along with a small amount of hyaluronic acid

SUMMARY

Pig umbilical cord was found to be nearly as rich a source of hyaluronic acid as human umbilical cord The products derived from pig cord contained approximately the same proportion of nitrogen, acetyl, and glucosamine as human cord products and were also hydrolyzed by the enzyme

hyaluronidase Hyaluronic acid preparations from pig cord were nitrated and acetylated in the same manner, and inhibited the hyaluronidase-hyaluronic acid system to about the same extent as similar inhibitors made from human cord preparations In comparing the hyaluronic acid preparations from pig umbilical cord with those from human cord, the essential difference was in the relative viscosity The pig cord products were appreciably less viscous

Acetone storage of pig cords prior to processing enhanced the quality of the hyaluronic acid obtained and simplified the extraction and purification procedures, although the introduction of a boiling water treatment into the extraction procedure offered no particular advantage

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CHEMISTRY OF THE CHICK EMBRYO

VI ACCUMULATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE*

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Despite voluminous literature describing the chemical changes which accompany development of the chick embryo, there are few quantitative data on the occurrence of respiratory and glycolytic enzymes and coenzymes Since these catalysts are concerned with reactions which may be available for the processes of growth and differentiation, more knowledge of their activities during the incubation period is desirable

The functional existence, in early chick embryos, of DPN and of a glycolytic system requiring DPN (diphosphopyridine nucleotide, coenzyme I) has been denied by Needham and Lehmann (10) However, Meyerhof and Perdigan (8) were able to demonstrate phosphorylative glycolysis and DPN activity in 4 to 9 day chick embryos Novikoff, Potter, and LePage (11) extended these observations to the estimation of the amounts of typical intermediates in 3 to 10 day embryos, the demonstration of phosphorylative glycolysis in extracts, and the existence of significant quantities of DPN in extracts None of the data is sufficiently extensive for the evaluation of the accumulation rate of DPN in the developing chick The present report presents the results of the estimation of DPN in about 250 chick embryos ranging in age from 2 to 19 days

EXPERIMENTAL

Test System—The method of Myrbäck (9) was selected as the most suitable for this study Dried brewers' yeast, after being washed with water, in the presence of hexose diphosphate, glucose, manganese, and phosphate ions produces carbon dioxide only if DPN is also present The rate of carbon dioxide production is strictly proportional to the DPN concentration over a considerable range This method is both specific and sensitive and the rate of reaction is conveniently followed in standard Barcroft-Warburg respirometers The test system adopted for use in the present work was as follows 300 mg of apozymase (washed yeast preparation), 0 3 cc of 10 per cent sodium hexose diphosphate, 0 2 cc of 20 per cent glucose, 0 1 cc of 16 per cent $MnCl_2 \cdot 6H_2O$, 1 5 cc of 0 10 M phosphate, pH

* Aided by a grant from the Ella Sachs Plotz Foundation

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6 5, 0 9 cc of DPN solution in 0 1 M phosphate, pH 6 5, gas phase, air, temperature, 30°, fluid volume, 3 0 cc

The apozymase was prepared from "2040" dried brewers' yeast (Fleischmann) by the method of Kensler, Dexter, and Rhoads (4) Drying was effected by vacuum distillation of the water from the shell-frozen yeast suspension The dried apozymase was found to be stable for several months if kept dry and cold

Hexose diphosphate was prepared by the method outlined by Robinson and Morgan (13)

DPN was prepared by the method of Williamson and Green (15) Our preparation was compared in the test system with two samples of DPN obtained from different sources and of different but known purities The purity of our sample as indicated by these experiments was 80 per cent Both pentose determinations (7) and phosphorus determinations (1) were consistent with 81 per cent purity Allowance was made for 80 per cent purity in the calculations

Under the conditions of our experiments the extra carbon dioxide produced per hour was proportional to the DPN added in the range of 0 to 15 γ All determinations were done in this range Our apozymase and DPN gave 65 per cent of the carbon dioxide production rate observed by Meyerhof and Perdigan (8) per microgram of DPN It remained unchanged throughout the experimental period

Embryological Material—Fertile eggs, from Rhode Island red hens, collected in trap nests and received in 2 dozen lots within 48 hours of laying were incubated in a commercial incubator at 38 5° and 50 to 60 per cent relative humidity The eggs were rotated twice daily and removed for analysis in groups of four

Preparation of Tissues—The grinding of animal tissues liberates a heat-labile system which rapidly destroys DPN In minces of chick embryo tissues this inactivation (8) proceeds rapidly even at temperatures just above freezing Since the enzyme system responsible for this inactivation is destroyed by heating at 80° (3), the following procedure for reducing the embryos to a homogeneous suspension was adopted The embryos were removed from the egg and freed from extraembryonic membranes After superficial drying and accurate weighing, the smaller embryos (3 to 1000 mg) were dropped whole into 20 volumes of 0 1 M phosphate buffer, pH 6 5, and heated for 5 minutes at 80° They were then cooled rapidly in a cold water bath and ground in a glass homogenizer as described by Potter and Elvehjem (12) The large embryos were dropped into 20 volumes of buffer previously heated to 80–90° after their weights had been obtained They were then cut into small pieces with scissors while in the hot solution This mixture was maintained at 80° for 5 minutes After rapid cooling it was homogenized as described above or in a Waring blendor if the bones

TABLE I

Recovery Experiments and CO_2 Production with Varying Amounts of Tissue

DPN added, γ	0	7 5	0	5*	7 5*	0
Tissue added, *mg*	0	0	34	34	34	102
CO_2 production per hr, *c mm*	137	424	377	567	666	857
Net CO_2 production per hr, *c mm*		287	240	430	523	720
DPN found, γ			6 3	11 2	13 7	18 8
Calculated, γ				11 3	13 8	18 9
Recovery, %				99	99	99

* Incubated 0 5 hour with heat-inactivated tissue

TABLE II

Accumulation of DPN in Chick Embryos

No of embryos in group	Average weight	Average age calculated	Average DPN
	mg	*days*	*γ per gm wet weight*
14	3 06	2 06	130
12	11 4	2 62	140
8	18 9	2 86	133
8	28 5	3 10	144
7	74 5	3 65	109
12	122 0	4 06	111
10	204 3	4 86	105
10	292	5 37	122
10	548	6 40	133
10	680	6 78	140
10	886	7 29	148
10	1,041	7 64	162
9	1,229	8 00	151
10	1,386	8 26	144
10	1,803	8 89	132
10	2,200	9 40	121
10	2,896	10 14	137
10	3,526	10 72	142
10	4,514	11 46	148
10	6,409	12 65	158
10	9,092	13 93	171
10	11,350	14 79	177
10	12,970	15 35	192
10	15,580	16 18	166
10	21,200	17 58	155
10	27,100	18 84	125

were so large as to interfere with adequate grinding in the glass homogenizers For analysis 0 9 cc portions of these 4 8 per cent suspensions were pipetted into the Warburg vessels for DPN estimation

Results

Table I demonstrates the almost complete recovery of DPN added to 10 day-old embryo tissue (heated as above) and incubated with it for half an hour at 37°.

It will be noted that the control vessel to which no DPN was added also produced some carbon dioxide. This was apparently due to residual DPN or other factors in the yeast and was subtracted as a blank from each determination. With each set of four determinations, the blank carbon dioxide production and the CO_2 per hour per microgram of DPN were determined.

Determinations of DPN were made on a total of 252 embryos. In all but the very early ages the estimations were made on individuals. The embryos were arranged in a series according to weight and averaged in groups of about ten, both as to the DPN content and to the age as calculated from the average weight. The rationale of using a calculated age is discussed by Levy and Palmer (5).

Table II summarizes this data showing the relationship between DPN concentration and calculated age.

DISCUSSION

Needham and Lehmann (10) reported that they were unable to demonstrate the presence of DPN in embryonic chick tissue. On the basis of this finding and from other considerations, they proposed that glycolysis in chick embryo tissue does not proceed by way of the usual glycolytic system as found in adult mammalian tissue. From the present study, it is apparent that although DPN is present in low concentration as compared to mammalian tissue, it is by no means absent. Our values are in the range reported by Meyerhof and Perdigan (8) (1.5 to 3.0 mg. per gm. of dry weight, equivalent to 120 to 240 γ per gm. of wet weight) and 3 to 6 times those reported by Novikoff, Potter, and LePage (11) (4 to 6 γ per 100 gm., equivalent to about 35 γ per gm. of wet weight). The agreement between the data of Meyerhof and Perdigan (8) and ours is not surprising, since the methods used were not significantly different. On the other hand, Novikoff, Potter, and LePage (11) used malic dehydrogenase as the test system. However, the system was also calibrated with DPN and since both methods are comparative this could not account for the 3- to 6-fold difference in the values found. It seems most likely that the preparation of the samples for analysis is accountable. The lower values were obtained by a method which depends on freezing and cold to prevent enzymatic destruction of DPN. It seems probable that in the preparation or test period, or both, some DPN was destroyed by the surviving enzyme in the tissue extracts.

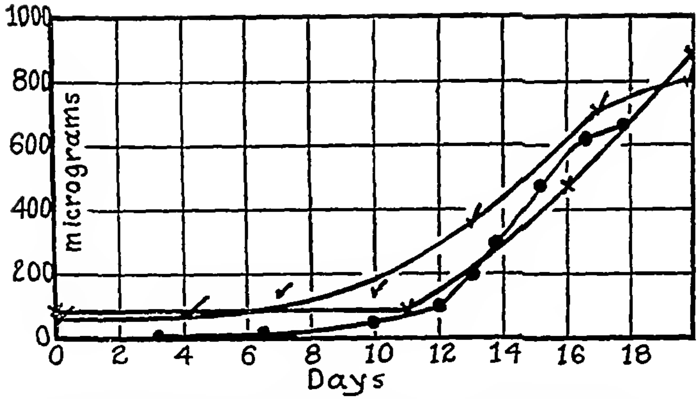

FIG 1 Nicotinic acid per egg and DPN per embryo during growth ✓ nicotinic acid per egg (14), × nicotinic acid per egg (2), ● nicotinic acid equivalent to the DPN per embryo in Table II

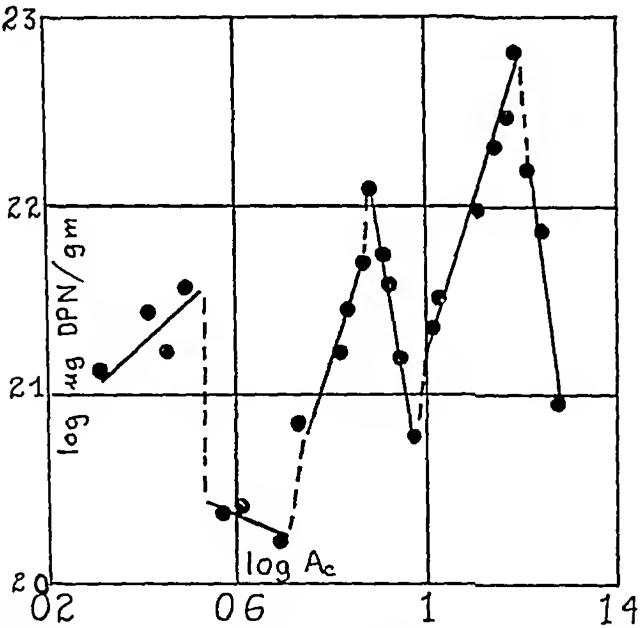

FIG 2 Relative accumulation diagram of chick embryo DPN A_c is the age in days calculated from the weight Each straight line segment (phase) conforms to the equation $\log Q/W = (i_Q - i_W) + (a_Q - a_W) \log A_c$ The dashed portions are at interphases and are less certain The values of i_Q and a_Q for DPN are given in Table III as the phase parameters

It is of interest to compare the amount of nicotinic acid bound as DPN in the embryo with the quantity of nicotinic acid in the whole egg contents The data of Handler and Dann (2) and of Snell and Quarles (14) both indicate an active synthesis of nicotinic acid by the embryo Fig 1 shows the total nicotinic acid in the egg as determined by these workers Also shown is the nicotinic acid equivalent to the DPN found in the present study Although the breed of chick is different in each curve, the comparison shows that in the later stages of development a large part of the total nicotinic acid is to be found in the DPN The data suggest that the amount of nicotinic acid synthesized by the chick is limited chiefly to the requirement for the formation of this coenzyme Since this substance is part of the functional structure of the chick (rather than a stored material), it seems unlikely that the availability of more nicotinic acid or its precursors would have any effect on the amount of DPN formed during development We therefore cannot conclude that the amount of DPN formed is limited by the chick's ability to synthesize nicotinic acid

TABLE III

Phase Parameters for Accumulation of DPN in Chick Embryo

Log micrograms of DPN = $i_Q + a_Q$ log age in days See Fig 2

Interphases, *days*	(2 0)*		3 5		4 5		7 6		9 6		16		(19)*
a_Q		5 29		5 21		4 34		1 78		4 36		1 70	
i_Q		−2 02		−2 08		−1 58		0 62		−1 64		0 37	

* The figures in parentheses indicate the beginning or end of the period of data

Fig 2 is a "relative accumulation diagram" (5) on which the logarithms of DPN concentrations in micrograms per gm of wet weight of embryo are plotted against the logarithms of the corresponding ages in days We have found such plots useful in demonstrating the periods of apparent regularity of growth implicit in a linear relationship between the ordinates of logarithms of amount of material (in this case DPN) and logarithms of age These periods of regularity (phases) are terminated at times (interphase times) when an abrupt adjustment of constants is necessary to describe the data Six phases separated by five interphases are evident in Fig 2 From the slopes and intercepts of the phase lines and the appropriate weight-age relationships (5) the "phase" parameters of Table III were obtained It is noted that the interphase at 4 5 days has been found in all the accumulation data so far obtained, that 7 6 days is an interphase in cytochrome oxidase accumulation (7 9 days) (6), that 3 5 and 9 6 days are interphases in dipeptidase accumulation (3 6 and 9 6 days), and that 16

days is an interphase in nitrogen accumulation (5) The interpretation of these coincidences remains for the future

SUMMARY

1 The diphosphopyridine nucleotide contents of chick embryos have been estimated from 2 to 19 days incubation

2 The data indicate that the coenzyme is present in the earliest embryos and contains most of the nicotinic acid of the egg in older embryos

3 The accumulation of the coenzyme follows the pattern of periods of constant multiplication rate in logarithmic time units previously shown to hold for other materials

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THE MEASUREMENT OF TURNOVER OF THE VARIOUS PHOSPHOLIPIDES IN LIVER AND PLASMA OF THE DOG AND ITS APPLICATION TO THE MECHANISM OF ACTION OF CHOLINE

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Experimental evidence presented in earlier reports failed to support the thesis that phospholipides are instrumental in the transport of fatty acids between organs (1) Another opportunity for examining this question presented itself in connection with the lipotropic action of choline, which has been attributed to a stimulation in the metabolism of phospholipides such as to increase the transport of fatty acids out of the liver (2)

The observations of Entenman *et al* on dogs injected with P^{32} demonstrated that choline increases the specific activity of the choline-containing phospholipide P of plasma and of liver (3) Such increases in liver and plasma could either result from an increase in the turnover of the phospholipides *per se* or merely reflect an increase in the specific activity of their precursors unassociated with an actual increase in turnover of these phospholipides To differentiate between these two possibilities, it became necessary to calculate the turnover rates of liver and plasma phospholipides This has been attempted in the present investigation

To calculate the turnover of a compound, one has to know the specific activity-time relations of that compound and of its immediate precursor (4) This measurement can be made for plasma lecithin and for plasma sphingomyelin because it has been shown by Fishler *et al* that the immediate precursors of these plasma phospholipides are their corresponding liver phospholipides (5)

So far as the liver phospholipides are concerned, however, their precursors have not been established with certainty, and hence the determination of the turnover rates of liver phospholipides is rendered difficult if not impossible

Several investigators (6-8) have attempted to identify the phosphorus-containing precursors of liver phospholipides Thus Flock and Bollman have shown that after the injection of P^{32} into birds the specific activity of liver glycerophosphate P is higher than that of liver phospholipide P, and

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from this observation it is conceivable that the former is the precursor of lecithin and cephalin in the liver (7) It therefore became of interest to us to determine whether in the dog glycerophosphate satisfies more specific requirements proposed earlier for identification of a precursor (4)

The separation of glycerophosphate from other acid-soluble P compounds by means of its barium salt is a laborious procedure and is open to criticism Kurssanov (9), for example, observed that, at the pH at which inorganic phosphate is completely precipitated by barium, most of the glycerophosphate is also removed from solution He also found that after the heating of glycerophosphate with 10 per cent NaOH for 3 hours or more only 20 per cent of the phosphorus was split off, whereas the same treatment caused the loss of 80 per cent of the phosphate of hexose monophosphate He

Diagram 1

Phospholipides in petroleum ether, treat with magnesium oxide and centrifuge

All phospholipides adsorbed, elute with methanol

Ppt = MgO and cephalins, heat with KOH and centrifuge

Supernatant = cephalin P

Supernatant = choline-containing phospholipides, hydrolyze with 1 N KOH at 37° and acidify with TCA

Ppt = sphingomyelin P

Filtrate = lecithin P

therefore suggested that the differential hydrolysis of phosphorus-containing compounds by strong alkali might provide a method for the separate determination of these compounds

As a result of these findings we undertook to prepare an acid-soluble fraction that was highly concentrated with respect to glycerophosphate, in the expectation that the specific activity of this fraction would resemble closely the specific activity of glycerophosphate

EXPERIMENTAL

Methods

Separation of Phospholipides into Lecithins, Cephalins, and Sphingomyelins (*Diagram 1*)—The phospholipides of liver and plasma were first extracted with alcohol and ether and finally dissolved in petroleum ether by a procedure described elsewhere (10) Choline-containing and non-choline-containing phospholipides were then separated after the manner of Taurog *et al* (11) In this procedure the choline-containing phospholipides were

eluted with methanol, whereas the cephalin fraction remained with the magnesium oxide. In the case of plasma phospholipides such a separation was not necessary, since it was shown by Taurog *et al.* that the phospholipides of dog plasma consist almost entirely of lecithins and sphingomyelins (12).

The choline-containing phospholipides were next suspended in 1 N KOH and incubated at 37° for 24 hours, so as to separate lecithin and sphingomyelin P by the method of Schmidt *et al.* (13). An aliquot of the trichloroacetic acid filtrate (TCA, Diagram 1), obtained by the method of Schmidt *et al.*, was used for the determination of lecithin P^{32}. Another aliquot of this filtrate was oxidized with perchloric acid and its P^{31} content determined colorimetrically (14).

Immediately after the filtration that separated lecithin and sphingomyelin phosphorus (Diagram 1), the precipitate was washed with 10 per cent trichloroacetic acid and was then removed from the filter paper with a few cc. of water. The precipitate was next dissolved by adding to it 1 cc. of 10 per cent NaOH and heating to 50-60°. An aliquot was then taken for the measurement of its P^{32} content and the rest of the solution ashed with perchloric acid for the determination of P^{31}.

To determine the specific activity of the cephalin P, 1 to 2 gm. of the MgO (which contained the cephalin phosphorus) were heated overnight on a steam bath with 25 cc. of 1 N KOH. After centrifugation the supernatant was transferred to another vessel and the MgO washed once with 20 cc. of distilled water. More than 90 per cent of the P^{32} adsorbed on the MgO was recovered in the KOH phase. The P^{32} and P^{31} content of the KOH solution was determined as described above for lecithin.

Test for Separation of Sphingomyelin P from Lecithin P—Schmidt *et al.* tested their method for the selective saponification of phospholipides on known mixtures of pure lecithin, cephalin, and sphingomyelin (13). For our purpose it seemed advisable to investigate the separation of lecithin and sphingomyelin phosphorus in dog plasma, a fluid that contains only these two phospholipides.

A sample of dog plasma was divided in two portions. One was treated in the usual way with alcohol and ether to remove its phospholipides. The extracted phospholipides were then subjected to hydrolysis at 37° and sphingomyelin determined as the difference between total P and lecithin P.

The other portion of the plasma was treated with colloidal iron according to the method of Folch and Van Slyke (15). In this way an extract of plasma phospholipides was prepared free of non-lipide nitrogen. The N:P ratio of this extract was then used to calculate the sphingomyelin content of the phospholipides. The results of the two procedures are compared in Table I.

It should be noted here that the procedure of Schmidt *et al* is based on the determination of a small difference between the total phospholipide P and the lecithin P, hence the percentage error in the values for sphingomyelin P obtained by this method may be much larger than the errors in the individual P determinations The determination of the specific activity of sphingomyelin is, however, not subject to this error, since the ratio of P^{32} P^{31} determined in the same solution is not affected by losses of P during transfers or washings

Separation of Acid-Soluble Phosphorus Fraction into Alkali-Stable and Alkali-Hydrolyzable Portions—About 10 gm of ground liver were homogenized with 10 cc of 10 per cent trichloroacetic acid (TCA, Diagram 2) and the mixture centrifuged The supernatant was made up to a volume of 50 cc The trichloroacetic acid extract was next neutralized with 16 N NaOH 5 gm of NaOH (pellets) were then added to each tube and the mixture heated

TABLE I

Comparison of Sphingomyelin Content of Plasma by N P Ratios and by Method of Schmidt et Al

Sample No	Sphingomyelin as per cent of total phospholipides	
	From N P	Method of Schmidt *et al*
1	18	21*
2	28	20†
3	12	14†

* Average of two determinations
† Average of three determinations

in a boiling water bath for 3 hours After it had cooled, the hydrolysate was neutralized with concentrated HCl 10 cc of a magnesia mixture[1] and 7 cc of 28 per cent aqueous NH_3 were then added in this order The magnesium ammonium phosphate which formed was allowed to settle overnight After filtration and thorough washing with 3 per cent NH_3, the phosphate was dissolved in 1 2 N HCl Filtrates A and B (Diagram 2) were used for the determination of the specific activity of the alkali-stable and of the alkali-hydrolyzable P, respectively

[1] The magnesia mixture consisted of 55 gm of $MgCl_2$ $6H_2O$, 70 gm of NH_4Cl, 650 cc of H_2O, and 350 cc of 10 per cent aqueous NH_3 Kurssanov (9) showed that in the precipitation with the magnesia mixture the final concentration of ammonia is very important Insufficient concentrations of ammonia were shown to result in incomplete precipitation of the inorganic phosphate, whereas high doses of ammonia brought about the precipitation of glycerophosphate In our experiments we used the optimal aqueous NH_3 concentration proposed by Kurssanov for the separation of inorganic phosphate from glycerophosphate

Test for Exchange Reactions during Fractionation Procedure—Since the glycerophosphate was heated in the presence of strong alkali, it seemed possible that the phosphorus moiety might exchange with the phosphate of the alkali-hydrolyzable fraction. Chargaff (16) has already presented data to show that such an exchange reaction does *not* occur in an acid medium.

In order to test whether exchange took place under the conditions of the procedure described above, glycerophosphate containing 4 mg. of P was heated for 5.5 hours on the steam bath with 4 mg. of radioactive phosphate

DIAGRAM 2

10 gm. liver, homogenize in 10% TCA and centrifuge

- Ppt. = proteins, etc.
- Supernatant = acid-soluble P, hydrolyze for 3 hrs. with 10% alkali, neutralize
  - Add magnesia mixture and filter
    - Ppt. = inorganic P and all organic P hydrolyzed to inorganic P
      - Dissolve in HCl and filter
        - Filtrate B = alkali-hydrolyzable P
    - Filtrate A = alkali-stable P
      - Mostly glycerophosphate

in a 10 per cent NaOH medium. After neutralization and addition of the magnesia, the sample was kept cold overnight in order to remove all inorganic phosphate. Almost all the P of the filtrate was found to consist of glycerophosphate as measured by Burmaster's method (17). Only 0.03 per cent of the P^{32} was found in this fraction. This means that no exchange had taken place under conditions of our analytical procedure.

Preparation of Radioactive Glycerophosphate—250 gm. of liver were obtained from a dog which had received intravenously radioactive phosphate 16 hours previously. Its phospholipides were first isolated as described elsewhere (10) and then redissolved in petroleum ether and reprecipitated with acetone.

400 cc of barium hydroxide containing 25 gm of $Ba(OH)_2 \cdot 8H_2O$ were added to 4 gm of phospholipides which had been dissolved in 200 cc of hot methanol, thorough shaking was employed during the mixing to insure emulsification of the phospholipide Most of the methanol was evaporated by heating the mixture for 2 hours on the steam bath About 4 cc of concentrated H_2SO_4 were next added to make the solution acid as indicated by brom-cresol green and the precipitate which formed filtered off The filtrate was shaken three times with equal volumes of petroleum ether containing 5 per cent chloroform to remove unhydrolyzed phospholipides and other fatty materials The petroleum ether washings contained practically no radioactivity According to Burmaster's method of measurement (17), practically all of the phosphorus in the water phase was present as glycerophosphate

TABLE II

Specific Activities of Phospholipides and Acid-Soluble Fractions in Two Different Lobes of Liver

Dog No	Fraction	Lobe A	Lobe B
22	Lecithin	11 1	11 2
	Cephalin	8 74	8 70
	Sphingomyelin	5 97	6 09
6	Alkali-stable	14 2	14 7
	Alkali-hydrolyzable	14 8	14 9

Animal Preparations

Normal and fat-fed dogs were used in this investigation The former were fed a high protein (lean meat) diet for several weeks before the experiment The latter received a high fat, low protein diet (its composition has been recorded elsewhere (3)) for 4 days before being used All dogs were first fasted for 24 hours and then lightly anesthetized with nembutal and kept so for the duration of the experiment Several samples of liver were excised as described in a previous report (3) Each sample was ground thoroughly, one aliquot was transferred to 95 per cent alcohol for determination of its phospholipide content, whereas another sample was homogenized in 10 per cent trichloroacetic acid for the determination of the two acid-soluble P fractions (Diagram 2)

Since samples of liver were taken from various lobes, it became necessary to inquire into the degree of variation of all P fractions found at a single time in the lobes of the liver This has already been done for two phospholipide fractions (3) The results presented in Table II for two separate lobes lend further support to the view that the specific activity of a phosphorus compound measured in a small sample of liver is a reliable index of the specific activity of that compound in the whole liver

Results

Normal Dogs—Biopsy samples of the liver (10 to 15 gm) were excised from Dog A at intervals of 3, 5, 9, and 12 hours, and from Dog B at 12.5, 16.5, and 21 hours after the administration of P^{32}. The samples were analyzed for total phospholipide P, alkali-stable P, and alkali-hydrolyzable acid-soluble P. The values for the specific activities of these three fractions, expressed as percentages of the injected P^{32} per mg. of phospholipide P, are plotted in Fig. 1.

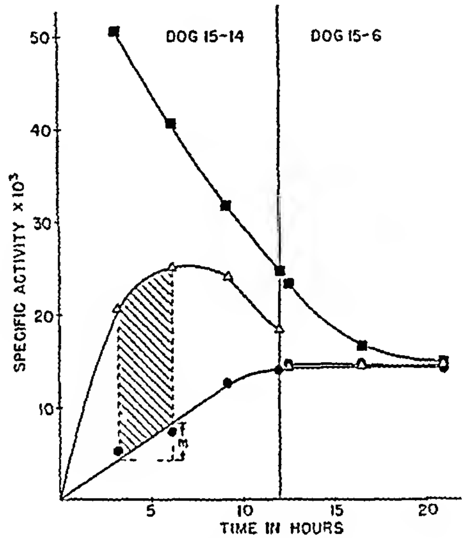

Fig. 1. Specific activity time relation of liver alkali-hydrolyzable P (■), liver alkali stable P (△), and liver phospholipide P (●).

It was pointed out by Zilversmit *et al.* (4) that the following relations exist between a compound and its precursor: (1) during the early intervals after a single injection of P^{32}, the specific activity of the precursor is higher than that of the compound, (2) at the time when the specific activity of the compound is maximal, it is equal to that of the precursor, (3) thereafter the specific activity of the precursor is lower than that of the compound. These criteria for a precursor hold only for single compounds. Although, as judged by these criteria, both alkali-stable and alkali-hydrolyzable phosphorus could be considered precursors of liver phospholipide phosphorus, the latter should be dismissed from consideration because its phosphorus is derived from several different phosphorus-containing compounds. The alkali-stable fraction, however, consists largely of glycerophosphate, and our main interest was to test whether this compound satisfies the precursor requirements.

It should next be pointed out that the fraction designated phospholipide consists of several compounds We therefore proceeded to investigate the specific activity-time relations between each of the acid-soluble fractions and individual types of phospholipides, namely, lecithins, cephalins, and sphingomyelins The results obtained on three dogs (Nos 22, 36, and 56) are shown graphically in Figs 2 to 4 Dogs 22 and 36 were fed the lean meat diet, whereas Dog 56 received the high fat diet

For all three dogs, the specific activities[2] of the lecithin fraction were higher than those of the other phospholipides Figs 2 to 4 show that the alkali-stable fraction meets all the requirements set forth above for a lecithin precursor

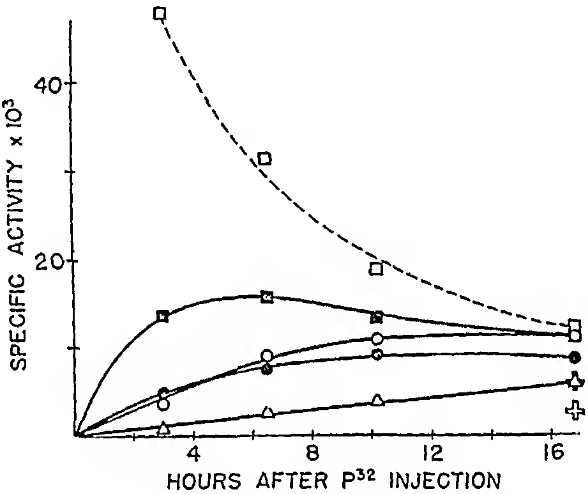

FIG 2 Specific activity-time relations (Dog 22) of liver alkali-hydrolyzable P (□), liver alkali-stable P (⊠), liver lecithin P (○), liver cephalin P (●), and liver sphingomyelin P (△), and the specific activities of plasma lecithin P (+) and plasma sphingomyelin P (open symbol)

Further evidence as to whether alkali-stable phosphorus is a precursor of liver lecithin phosphorus was sought in choline-treated dogs in which the shape of the liver lecithin specific activity curve is radically changed Maintenance of the relations outlined above between lecithin phosphorus and alkali-stable phosphorus under the new conditions provided by choline treatment would, of course, add support to the possibility that the alkali-stable phosphorus is the sought for precursor

Fat-Fed, Choline-Treated Dogs—Three dogs were fed high fat diets for 4 days and then fasted for 24 hours before the administration of 300 mg of choline per kilo of body weight The P^{32} was injected intravenously 30 minutes later The results on these three dogs are shown in Figs 5 to 7

[2] All specific activities are expressed as percentages of the injected P^{32} per mg of total P

A pronounced increase in the specific activities of alkali-hydrolyzable P, alkali-stable P, and lecithin P was observed in two of the three dogs Thus the maximum specific activity of the liver lecithin in the choline-treated

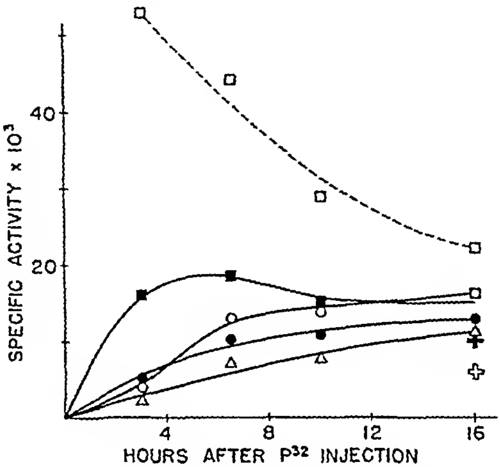

FIG 3 Specific activity time relations (Dog 36) of liver alkali hydrolyzable P (□), liver alkali stable P (■), liver lecithin P (O), liver cephalin P (●), and liver sphingomyelin P (△), and the specific activities of plasma lecithin P (+) and plasma sphingomyelin P (open symbol)

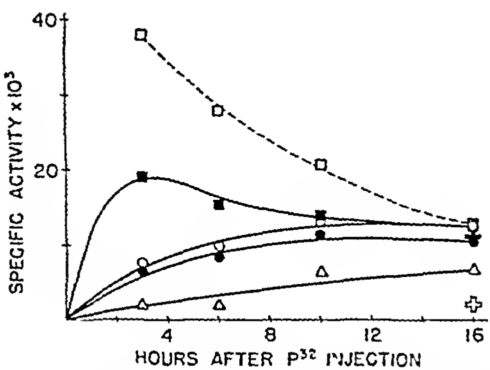

FIG 4 Specific activity time relations (Dog 56) of liver alkali hydrolyzable P (□), liver alkali stable P (■), liver lecithin P (O), liver cephalin P (●), and liver sphingomyelin P (△), and the specific activities of plasma lecithin P (+) and plasma sphingomyelin P (open symbol)

dog was twice that of the untreated dogs Despite the change in shape of the specific activity-time curve for liver lecithin that resulted from the administration of choline, the curve for alkali stable phosphorus retained features sufficient to characterize it as a possible precursor of liver lecithin

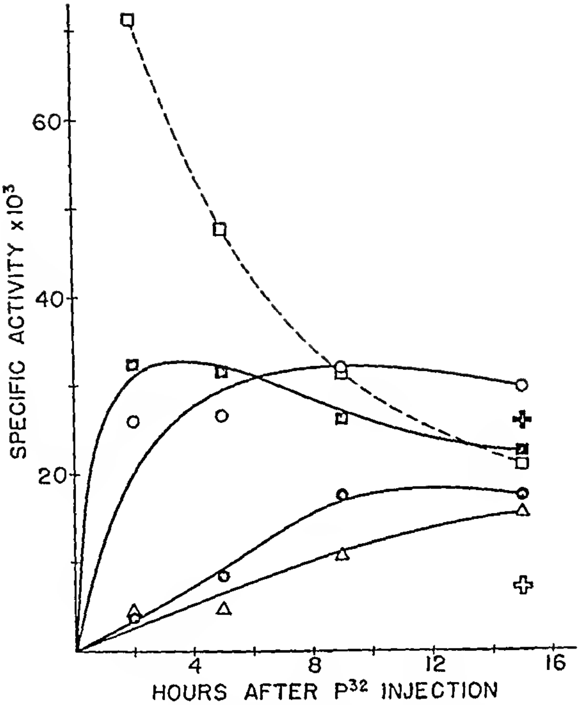

FIG 5 Specific activity-time relations (Dog 44) of liver alkali-hydrolyzable P (□), liver alkali stable P (⊡), liver lecithin P (○), liver cephalin P (●), and liver sphingomyelin P (△), and the specific activities of plasma lecithin P (✚) and plasma sphingomyelin P (open symbol)

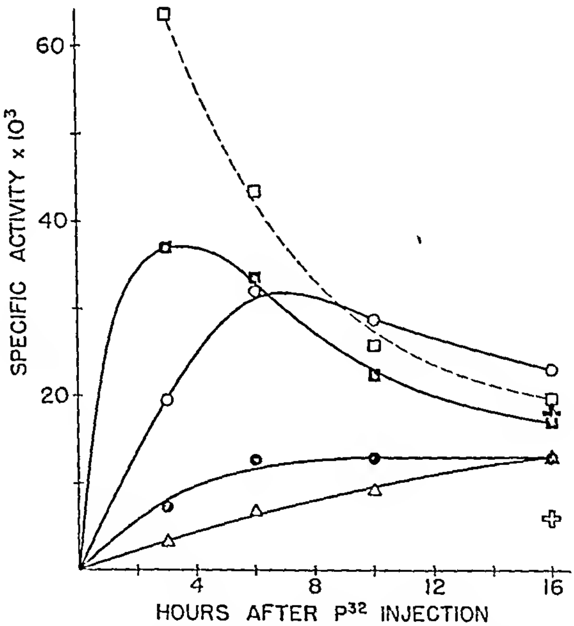

FIG 6 Specific activity-time relations (Dog 45) of liver alkali-hydrolyzable P (□), liver alkali-stable P (⊡), liver lecithin P (○), liver cephalin P (●), and liver sphingomyelin P (△), and the specific activities of plasma lecithin P (✚) and plasma sphingomyelin P (open symbol)

Calculation of Turnover Time of Liver Lecithin

From theoretical considerations presented elsewhere (4) it was concluded that a measure of the turnover time[3] of a phospholipide can be obtained from the specific activity-time curves of the phospholipide and its precursor The phospholipide turnover time, t_t, is obtained from the ratio of (1) the area bounded by these two curves between two time coordinates to (2) the increase in the phospholipide specific activity in that same time interval This procedure is illustrated in Fig 1 Thus,

$$t_t = \frac{\text{shaded area}}{m}$$

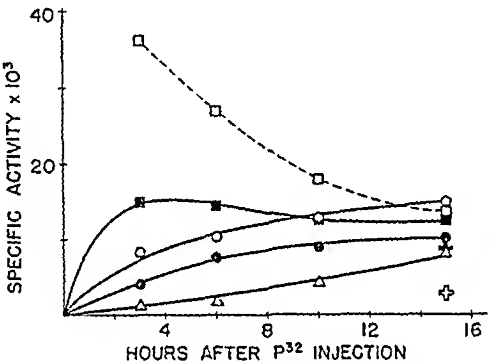

FIG 7 Specific activity time relations (Dog 57) of liver alkali-hydrolyzable P (□), liver alkali stable P (■), liver lecithin P (○), liver cephalin P (●), and liver sphingomyelin P (△), and the specific activities of plasma lecithin P (+) and plasma sphingomyelin P (open symbol)

For the measurement of t_t, it is necessary to use the specific activity at an early interval because (1) it is the time when the error in the determination of the difference between the specific activities observed at the beginning and end of the period could be expected to be minimal, since at the early intervals the specific activity of liver lecithin increases most rapidly, and (2) if deleterious effects of surgery occurred, they would be expected to be minimal at the early intervals Since we have no data on the shape of the curves up to the point when the first liver sample was taken, the calculation had to be applied to the area beyond this point We therefore chose the time interval between the taking of the first and second liver samples as most reliable for the calculation of t_t

t_t values for liver lecithin are recorded in Table III For reasons dealt

[3] The turnover time of a liver phospholipide is the time required for the turnover of an amount of liver phospholipide equal to that present in the liver

with above and in "Discussion" below, those based on the alkali-stable fraction as precursor are more reliable

The results in Table III clearly show that, regardless of which fraction is taken for the calculation, the t_t values for liver lecithin are decreased in the two dogs that responded to choline treatment Since the total lecithin in the livers of the choline-treated dogs remained normal, this means that liver lecithin is synthesized at a more rapid rate in the choline-treated than in the normal dog

TABLE III

Turnover Time of Liver Lecithin*

	Dog No	Weight	Based on alkali stable specific activity	Based on alkali hydrolyzable specific activity
		kg	*hrs*	*hrs*
Normal	22	12	5 9	21
	36	13	3 6	15
	56	11	6 1	18
Choline-treated	44	11	1 8	8 7
	45	13	2 0	5 9
	57†	14	5 6	21

* All values are obtained from the areas and specific activities between the first and second points on the curve (i e , 3 to 6 hours)

† This dog failed to show an increase in the specific activities of liver or plasma phospholipides after the administration of choline 85 per cent of the dogs treated with choline showed marked increases in phospholipide specific activities Irregularities in the response of animals towards the lipotropic action of choline are known to occur

Calculation of Turnover Time for Plasma Lecithin and Sphingomyelin

The specific activities of these two plasma phospholipides were elevated in the choline-treated dog Since the liver is the source of plasma phospholipides, this means either (1) that the turnover of plasma phospholipides had actually increased or (2) that the change in specific activities merely reflects similar changes in liver phospholipides unassociated with an actual increase in turnover of plasma phospholipides

In order to obtain the turnover time of plasma sphingomyelin we can make use of the fact that in nearly all cases the specific activity of liver sphingomyelin increases linearly with time Under such conditions the following relation has been shown to hold (4)

$$\frac{x}{r} = bt_t\left(\frac{t}{t_t} - 1 + e^{-t/t_t}\right) \tag{1}$$

in which x/r is the specific activity of plasma sphingomyelin at time t, b is the increase in specific activity of liver sphingomyelin per unit of time, and t_t is the turnover time for plasma sphingomyelin

If the specific activities of plasma sphingomyelin and of liver sphingomyelin at any one time are known (in our experiment we knew the values at the end of the experiment), then the turnover time of plasma sphingomyelin can be calculated from equation (1) The values so obtained are presented in Table IV

A calculation for turnover time of plasma lecithin is more complex, since the relation observed between the specific activity of liver lecithin and time is not simple In a previous communication the following equation was derived for the relation between the specific activity of phospholipide phosphorus and that of its precursor when the specific activity of the latter is kept constant (4)

TABLE IV

Turnover Time of Plasma Lecithin and Sphingomyelin*

	Dog No	Lecithin	Sphingomyelin
		hrs	*hrs*
Normal	22	11	12
	36	6	10
	56	10	13
Choline-treated	44	6	11
	45	12	10
	57		21

* For calculation see the text

$$\frac{x}{r} = a(1 - e^{-t/t_t}) \qquad (2)$$

where x/r is the specific activity of the phospholipide at time t, a the constant specific activity of its precursor, and t_t the turnover time of the phospholipide

In our experiment the specific activity of the phospholipide precursor did not remain constant (see Figs 1 to 7) A good approximation of t_t can be obtained when the average specific activity of liver lecithin (in this case the precursor) is substituted for a in equation (2) The average liver lecithin specific activity is calculated by dividing the area under the liver lecithin specific activity curve by the duration of the experiment The equation now becomes

$$\frac{\text{Plasma lecithin specific activity at end of experiment}}{\text{Average specific activity of liver lecithin}} = 1 - e^{-t/t_t}$$

The turnover times obtained in this way are presented in Table IV, the results of which clearly show that *choline does not significantly change the turnover rate of either plasma lecithin or sphingomyelin*

Even though the values for t_t of these two phospholipides were quite

similar, their turnover rates do, however, differ because the concentration of lecithin phosphorus in plasma is approximately 4 times as high as that of sphingomyelin phosphorus

DISCUSSION

In confirmation of earlier observations of Entenman *et al* (3), it is shown here that the specific activities of choline-containing phospholipides of liver and plasma are markedly elevated shortly after a single feeding of choline The present investigation brings to light, however, that nearly all of the rise is caused by lecithins Increases were also observed in liver sphingomyelin, but, since this lipide constituent constitutes only about 10 per cent of the liver's phospholipides, it contributes little to the rise in the specific activities of the choline-containing phospholipides of the liver

In those animals that responded to choline, the latter produced an increase in the calculated turnover of *liver* lecithins But in neither *plasma* lecithin nor *plasma* sphingomyelin was an increase in the calculated turnover rates observed after the administration of choline The increases in the specific activities of plasma phospholipide previously reported (18) must now be recognized as a reflection of similar increases of their precursors in the liver

The fact that choline stimulates lecithin turnover in the liver but not in plasma may have an important bearing on the mechanism of action of choline as a lipotropic agent *If the lipotropic action of choline operates through phospholipide metabolism, then the removal of liver fat under the influence of choline does not involve an increased transport of fat from liver to peripheral tissues via plasma phospholipides The evidence suggests rather that choline acts on the utilization of fat within the liver itself*[4]

Although the main purpose of this paper was to obtain a liver phosphorus fraction that would enable us to calculate phospholipide turnover in this tissue, it is nevertheless of interest to consider the composition of the alkali-stable fraction An analysis of this fraction for its glycerophosphate content was made by Burmaster's method (17)[5] In the three normal dogs,

[4] Platt and Porter (19) have reported that ethanolamine stimulates cephalin turnover to the same extent as choline stimulates lecithin turnover Since ethanolamine is not lipotropically active, these authors suggest that the stimulation caused by choline is due to a mass action effect and not to a lipotropic mechanism

[5] Micromethods for the accurate determination of glycerophosphate in the presence of other organic phosphates have not been developed The oxidation of glycerophosphate with periodate and the subsequent hydrolysis of the glycolaldehyde phosphate have been used extensively This method has been criticized, both for its unspecificity (LePage (20)) and its low recovery (86 5 per cent according to Leva and Rapoport (21)) For our purpose, however, the periodate method seemed suitable, since we were mainly interested in learning whether the greater portion of the alkali-stable fraction (Diagram 2) was made up out of glycerophosphate

66 to 75 per cent of the total P in the alkali-stable fraction was accounted for by glycerophosphate P, whereas 6 to 9 per cent consisted of inorganic phosphate. In the choline-treated animals the percentages for glycerophosphate were 54 to 61, whereas the values for inorganic phosphate were 2, 3, and 11 per cent. These results leave no doubt that the alkali-stable fraction of liver as determined here is rich in glycerophosphate. Since glycerophosphate is structurally an important moiety of the lecithin molecule, and moreover since the specific activity of the alkali-stable fraction satisfied the criteria for a precursor of lecithin, it is likely that glycerophosphate[6] is actually a precursor of liver lecithin.

In an attempt to obtain more direct evidence on the nature of the precursor of liver lecithin, a sample of radioactive glycerophosphate was prepared and injected into the portal vein of a dog at a constant rate during a period of 2.5 hours. At the end of this interval a liver sample was taken. The analyses showed that the specific activity of the inorganic phosphate in the liver was just as high as that of the alkali-stable fraction. It was therefore not possible, from this experiment, to draw more definite conclusions as to whether glycerophosphate is a precursor of liver lecithin.

Since Stetten (22) found that ethanolamine can be considered a precursor of choline, it may be noted that in the present experiments the specific activities of lecithin P in the liver were higher than those of cephalin P. This means that liver cephalin is not on the main pathway of lecithin synthesis and suggests that bound ethanolamine, in contrast to its free form, is not methylated to an appreciable extent.

SUMMARY

1. The specific activity-time relations of liver lecithin, liver cephalin, and liver sphingomyelin were compared with those of two acid-soluble fractions, namely alkali-stable and alkali-hydrolyzable phosphorus.

2. The specific activity-time relations of a liver fraction rich in glycerophosphate met the requirements for a lecithin precursor.

3. Choline increased the turnover of liver lecithin but not of plasma lecithin or of plasma sphingomyelin.

4. In view of these findings it is proposed that choline does not act by increasing fat transport via plasma phospholipides but rather by stimulating the utilization of fats within the liver itself.

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THE TURNOVER RATES OF PLASMA LECITHIN AND PLASMA SPHINGOMYELIN AS MEASURED BY THE DISAPPEARANCE OF THEIR RADIOACTIVE PHOSPHORUS FROM THE CIRCULATION

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Dog plasma contains only two types of phospholipides, namely lecithins and sphingomyelins (1) This is worthy of note, since plasma phospholipides originate in the liver (2), a tissue that synthesizes three types of phospholipides, cephalins as well as those containing choline The data obtained in the preceding paper (3) showed that lecithin is delivered to plasma at a much more rapid rate than sphingomyelin Since this measurement was based on a procedure that involved considerable surgical trauma of the liver, the values obtained could not be considered entirely accurate without further proof A measure of their turnover in plasma was therefore sought by a different method, *i e*, one that would cause no physiological disturbance This was accomplished by introducing labeled plasma phospholipides into the circulation of dogs and observing the rates of their disappearance from the blood stream (4) In contrast to this method, which measures the rate of disappearance of plasma phospholipides, the procedure used in the preceding report was based on the rate at which phospholipides are delivered by the liver to plasma Since in the steady state the amount of each phospholipide that enters the plasma must equal that which leaves, the two measurements should yield identical results The close agreement shown here for rates of turnover of each plasma phospholipide as measured by two widely differing methods lends support to the reliability of the data presented

EXPERIMENTAL

The methods used for the determination of lecithin and sphingomyelin P^{31} and P^{32} are described in the previous communication (3)

In Experiment 1 radioactive phospholipides were obtained from a donor dog that had received orally 4 millicuries of P^{32} *24 hours* before exsanguination Enough blood was obtained from this donor dog to permit an injection of 100 cc of plasma into each of three recipient dogs designated here as Dogs A, B, and C The data for the specific activities of lecithin and

* Fellow of the American Cancer Society, recommended by the Committee on Growth of the National Research Council

sphingomyelin phosphorus of plasma found at various intervals after the injection of radioactive phospholipides are presented in Figs 1 and 2

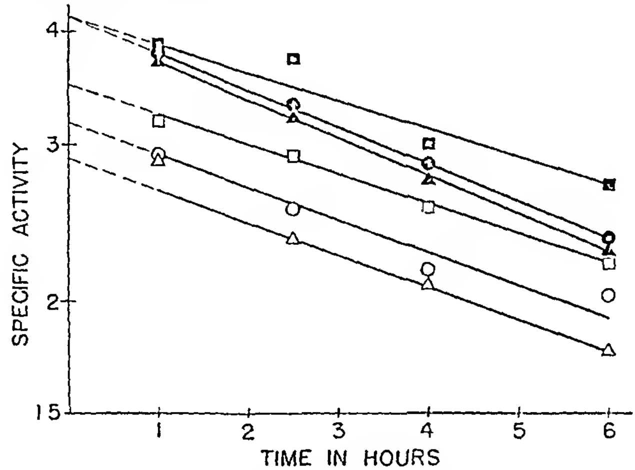

FIG 1 The disappearance of labeled lecithin from the circulation Dog A, Experiment 1 (○), Experiment 2 (**o**), Dog B, Experiment 1 (□), Experiment 2 (■), Dog C, Experiment 1 (△), Experiment 2 (▲)

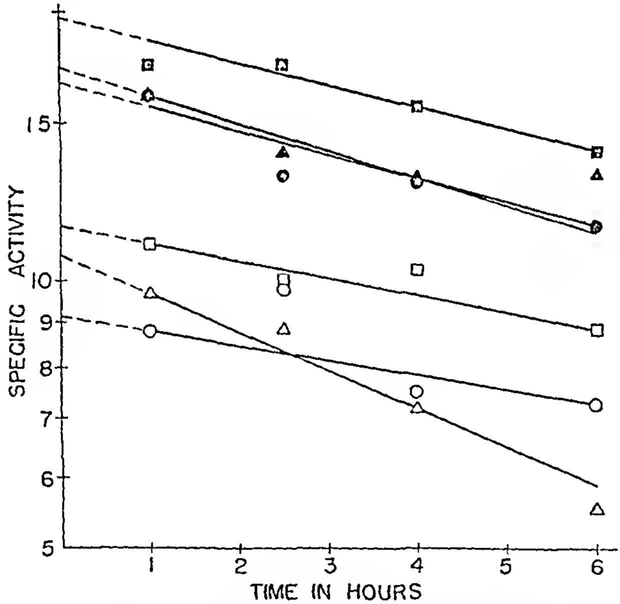

FIG 2 The disappearance of labeled sphingomyelin from the circulation Dog A, Experiment 1 (○), Experiment 2 (**o**), Dog B, Experiment 1 (□), Experiment 2 (■), Dog C, Experiment 1 (△), Experiment 2 (▲)

Calculation of Turnover Time and Turnover Rate

It has been shown in a previous communication (4) that

$$\frac{x}{r} = \frac{x_0}{r} e^{-\frac{p}{r}t}$$

where x is the amount of phospholipide P^{32} present in the entire circulating

fluid at time t, x_0 is the amount of phospholipide P^{32} present in the entire circulating fluid at zero time, r is the total amount of phospholipide P present in the entire circulating fluid, p is the rate of phospholipide turnover, and t is the time after injection of the labeled phospholipide

Thus x/r represents the specific activity of phospholipide P at time t Since r/p is equal to the time required for the turnover of an amount of phospholipide P equal to that present in the circulating fluid,

$$\frac{x}{r} = \frac{x_0}{r} e^{-t/t_t} \tag{B}$$

where t_t is the phospholipide turnover time

Table I

Turnover Times and Turnover Rates of Plasma Lecithin and Sphingomyelin

Dog	Experiment No	Weight	Lecithin P	Sphingomyelin P	Turnover time		Turnover rate	
					Lecithin	Sphingomyelin	Lecithin	Sphingomyelin*
		kg	*mg per cent*	*mg per cent*	*hrs*	*hrs*	*mg P per hr*	*mg P per hr*
A	1	7 9	10 6	2 7	12	27	4 3	0 5
	2	8 0	8 7	1 9	12	17	5 6	0 7
B	1	8 8	10 3	2 1	12	23	4 0	0 4
	2	8 9	11 3	2 7	10	17	4 4	0 8
C	1	7 9	10 1	2 0	13	10	4 3	1 0
	2	8 5	9 9	1 7	10	14	5 3	0 7

* The estimation of the total amount of sphingomyelin present in the entire circulating fluid has been made on the basis of the lecithin specific activity data Thus it is assumed that the circulating volumes for lecithin and sphingomyelin are identical

Thus,

$$\ln \frac{x}{r} = \ln \frac{x_0}{r} - \frac{t}{t_t} \tag{C}$$

In Figs 1 and 2 the specific activity of phospholipide P at zero time, *i e* x_0/r, can be read from the intersection of the curve with the y axis If we define the half time ($t_{\frac{1}{2}}$) as the time at which the phospholipide specific activity is exactly half the specific activity at zero time, then we can derive from equation (C) the relation

$$t_t = \frac{t_{\frac{1}{2}}}{\ln \frac{x_0}{x_{\frac{1}{2}}}} = \frac{t_{\frac{1}{2}}}{\ln 2} = 1\ 44\ t_{\frac{1}{2}}$$

The turnover time is thus easily determined from the half time of any one of the curves in Figs 1 or 2 The turnover rate can be calculated if the total amount of phospholipide P in the circulation (r) is known

x_0 is the total amount of phospholipide P^{32} present in the circulation at a time when none of it has yet been utilized and is therefore equal to the total amount of phospholipide P^{32} injected Dividing the amount of injected phospholipide P^{32} by the specific activity of the phospholipide P at zero time thus gives the total amount of phospholipide P present in the circulation (r)

The turnover rate, p, is then found from

$$p = \frac{r}{t_t}$$

The values for turnover times and turnover rates are recorded in Table I

The same dogs were used in Experiment 2 carried out 3 weeks later A new donor dog was used, however, it received orally 4 millicuries of P^{32} *48 hours* before it was bled The good agreement between the results of both experiments (Table I) shows that the turnover times are not influenced by variables in donor dogs and the time allowed for the syntheses of labeled plasma phospholipides

It is shown here that the turnover times for plasma lecithin are a little shorter than those for plasma sphingomyelin Since, however, the plasma of these dogs contained about 4 to 5 times as much lecithin as sphingomyelin, the absolute amounts of these plasma phospholipides turned over (*i e*, their turnover rates) differ considerably Thus, plasma lecithin is turned over at a rate which is more than 5 times as great as that of plasma sphingomyelin

SUMMARY

The rates of turnover of plasma lecithin and of plasma sphingomyelin have been measured in the dog The rate for lecithin is more than 5 times as great as that for sphingomyelin

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ESTIMATION OF ARGINASE ACTIVITY IN HOMOGENATES*

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The present procedures were developed prior to a study of the changes in arginase activity in epidermal carcinogenesis because none of the existing methods was found to meet the minimal requirements for a good assay (1) Recent advances in the determination of arginase activity of purified liver extracts (2) and in the knowledge of metallic activation of arginase (3) have served as the basis for the modifications described herein

EXPERIMENTAL

Basic Procedure for Determination of Arginase Activity—A 2 per cent homogenate of the tissue to be studied was prepared in ice-cold redistilled water in a ground glass homogenizer One aliquot of the above was mixed with an equal volume of a 0 1 M solution of $MnCl_2 \cdot 4H_2O$ and placed in a water bath at 50° in tubes which were tightly stoppered after temperature equilibrium had been established Samples were removed at the desired intervals for measurement of activity The tubes were shaken vigorously every 30 minutes The method for the measurement of arginase activity was essentially that described under "Photometric method" by Van Slyke and Archibald (2) The activity of the original homogenate was measured by adding 0 1, 0 2, or 0 3 ml of the homogenate or a suitable dilution thereof to 5 ml test-tubes containing 0 5 ml of arginine substrate to which 0 9, 0 8, or 0 7 ml of redistilled water or of 0 05 M $MnCl_2$ had previously been added The activities of the samples incubated with $MnCl_2$ at 50° were measured only in tubes containing the Mn^{++} ion The tissue samples were added after the substrate solutions had attained the temperature of the bath (38°) The pH of the mixture containing distilled water as the diluent was approximately 9 5, while that of the mixture containing $MnCl_2$ was approximately 9 2 No differences in activity were observed when samples of the original homogenates were added directly to the Mn-containing incubation mixture or when the homogenates were made 0 05 M with respect to $MnCl_2$ prior to the addition This is because the Mn^{++} was present in considerable excess The reaction was allowed to proceed for exactly 10 minutes and

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then stopped by the addition of 1 ml of 15 per cent metaphosphoric acid The urea content was measured in the manner previously described (2) A reagent blank was prepared by adding 1 ml of the acid to the substrate solution to which 1 ml of water or 1 ml of 0 05 M $MnCl_2$ had been added The Mn^{++} ion did not affect the blank value A tissue blank was also prepared by adding the metaphosphoric acid to the substrate solution prior to the introduction of 0 1 to 0 3 ml of the homogenate Liver homogenates were always diluted to 1 10 or 1 20 with redistilled water or with 0 05 M $MnCl_2$ just prior to the determination of activity

LIVER

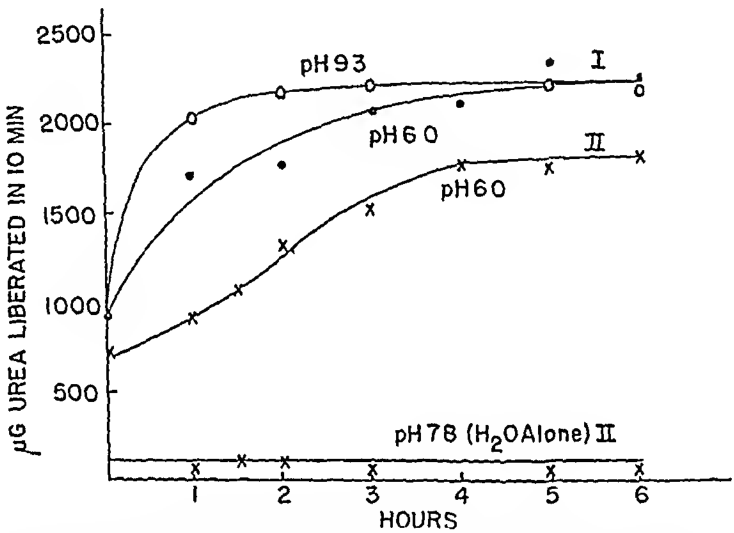

FIG 1 Change of activity with time of mouse liver homogenate incubated at pH 6 0 or 9 3 in 0 05 M $MnCl_2$ or in water alone at pH 7 8

Tissues Studied—Special attention was given to the applicability of the method to the determination of the arginase activity of the liver, kidney, and epidermis of the Swiss mouse and of a transplantable squamous cell carcinoma originally derived from the epidermis of a mouse by the application of methylcholanthrene (4) A survey was made of some of the other tissues The influence of the daily injection of 75 mg of arginine on the arginase activity of the liver, kidney, and epidermis of adult mice was also investigated

Results

Activation by Manganese—The results of the study of the time course of manganese activation at 50° are summarized in Figs 1 and 2 and Table I

Liver (Fig 1)—From the results of Experiment I it is seen that the maxi-

mal activity attained was the same whether the activation took place in an unbuffered solution at pH 6 0 or in 0 25 M glycine buffer at pH 9 3 The rate of activation was somewhat greater at the higher pH These findings are similar to those previously reported for purified beef liver arginase (3) The incubation in the unbuffered solution was employed routinely because of greater convenience and for the sake of maintaining uniformity with the procedure employed for the other tissues

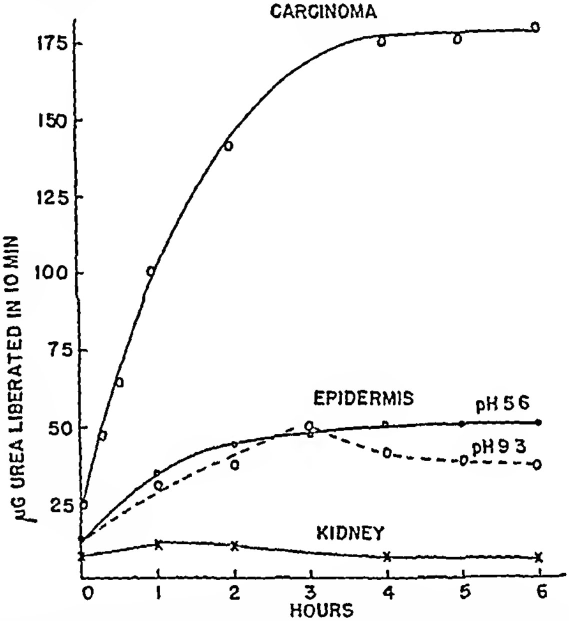

FIG 2 Activation of a homogenate of mouse epidermis in 0 05 M $MnCl_2$ at pH 5 6 and 9 3 and the activation of homogenates of squamous cell carcinoma and kidney in 0 05 M $MnCl_2$

Experiment II was performed on another liver sample The activity attained a maximal level in 4 hours and remained constant through 6 hours as in the comparable sample in Experiment I In both cases the maximal activity was approximately 2 5 times the activity of the freshly prepared homogenate when estimated in the presence of Mn^{++} ions Incubation with water alone at 50° produced no activation The unactivated homogenate had a 5-fold greater activity when the measurements were carried out in the presence of $MnCl_2$ than when no Mn^{++} ions were added

A 5 hour incubation period was employed for all routine assays In several experiments it was found that maximal activation was not achieved by heating to 58° for 20 minutes with $MnCl_2$, a procedure found suitable for purified arginase preparations (2)

Epidermis (Fig 2)—Incubation either at pH 5 6 or at pH 9 3 resulted in the same value for maximal activity This value was attained at the lower pH in 4 hours and remained virtually constant for 6 hours However, in the alkaline glycine buffer the activity declined after reaching the maximal level at 3 hours The activation of this tissue was therefore performed routinely in the unbuffered solution for 5 hours at 50° The maximal activity was approximately 5 times greater than that in the fresh homogenate when the measurements were performed in the presence of Mn^{++} ions

Carcinoma (Fig 2)—The activity for carcinoma reached a maximum 4 hours after the start of incubation in an unbuffered mixture and remained constant through 6 hours These findings are the same as for the two previously discussed tissues The extent of activation achieved was greater

TABLE I

Arginase Activity of Various Tissues of Mouse

The results are expressed in micrograms of urea liberated per mg of fresh weight under the standard assay conditions

Time of incubation		Tissue			
		Intestine	Lymph nodes	Leg muscle	Spleen
hrs					
0	No $MnCl_2$	130	1	1	1
0	With $MnCl_2$	111	1	1	1
1		6	1	1	1
2		2		1	1
3		4	3	1	1
4		2	3	1	1
6		14	4	2	1

in this tissue than in liver and epidermis, the ratio of final to initial activities in the presence of Mn^{++} ions being approximately 7·1 The 5 hour incubation was employed for all assays of carcinoma samples

Kidney (Fig 2)—The results of heating unbuffered homogenate of kidney in 0 05 M $MnCl_2$ at 50° are typical of those obtained in a number of similar experiments There was a small increase in activity after 1 hour and a slow rate of decrease thereafter The activity of kidney homogenates was therefore usually measured after 1 hour of incubation In a few instances no activation was observed

Other Tissues (Table I)—The addition of $MnCl_2$ to the substrate decreased the high activity of the homogenate of washed small intestine to 85 per cent of that found when no activator was added Most of the remaining activity was lost after incubation for 1 hour at 50° These results differ from those found for the other tissues studied and merit fur-

ther investigation Only slight activity was observed in lymph nodes, leg muscle, and spleen No activity was detected in heart, pancreas, stomach, brain, testes, whole blood, or thrice-washed erythrocytes in the presence or absence of added $MnCl_2$ or after various intervals of incubation with Mn^{++} It should be noted that the failure to find arginase activity in these latter tissues does not necessarily mean that they do not possess some arginase activity Different treatments and the utilization of higher tissue concentrations may be required for the demonstration

Proportionality of Arginase Activity to Quantity of Tissue—In Fig 3 are shown the results of experiments in which different quantities of maximally activated preparations of carcinoma, epidermis, and liver were tested under

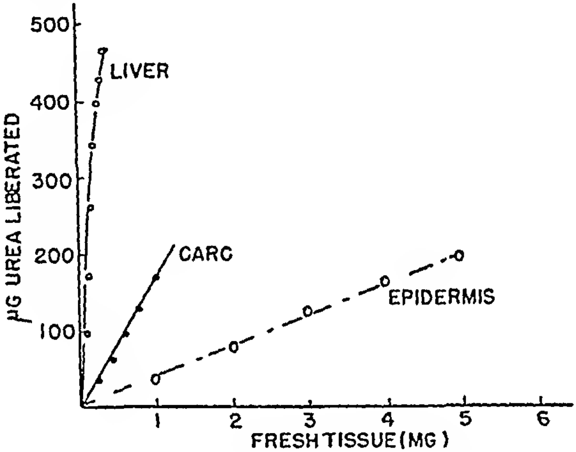

Fig 3 Proportionality of activity to quantity of tissue in liver, carcinoma and epidermis

the standard assay conditions In each case the activity was proportional to the tissue concentration when the quantities of tissue were suitably chosen Measurements of the activity of an unactivated homogenate of kidney made in the presence of Mn^{++}, Fig 4, show that also for this tissue the linearity holds true

Time Course of Liberation of Urea—The results of a kinetic study of the liberation of urea from arginine by maximally activated homogenates of epidermis, liver, and carcinoma are given in Fig 5 In each case 2 ml aliquots of a suitable dilution of the homogenate in 0 05 M $MnCl_2$ were added to a solution containing 8 ml of 0 05 M $MnCl_2$ and 5 ml of the arginine solution to give the final tissue concentrations indicated on the curves The reactions were carried out at 38° At various time intervals 1 ml samples were withdrawn and pipetted into tubes containing 0 67 ml of 15

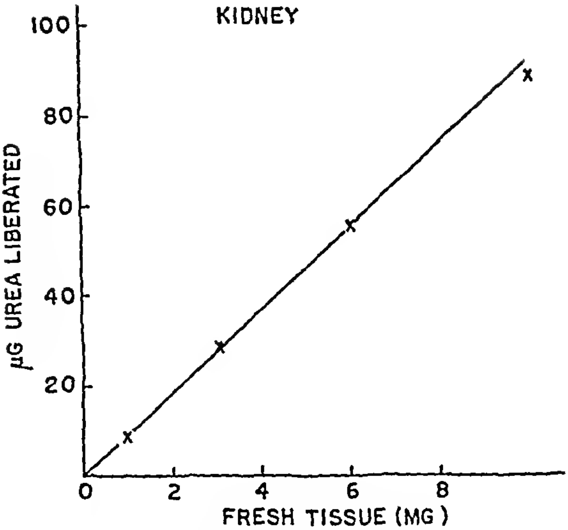

FIG 4 Proportionality of activity to quantity of tissue in kidney

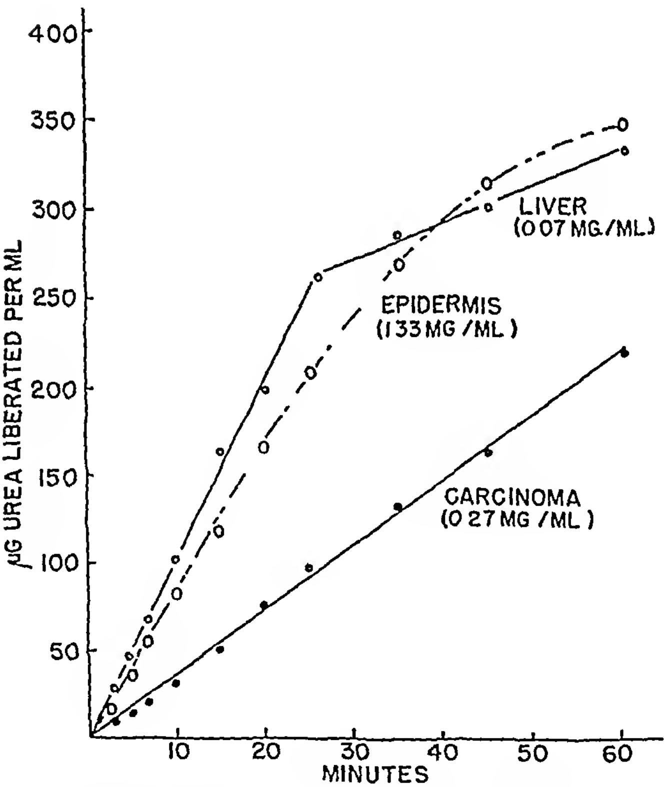

FIG 5 Time course of liberation of urea by maximally activated homogenates of liver, carcinoma, and epidermis

per cent metaphosphoric acid After centrifugation 1 ml samples of the supernatant fluid were employed for urea determination

For each tissue the reaction rate was constant over considerably longer periods than the 10 minutes employed in the standard assay procedure.

Typical Assay Results (Table II)—An increase in the arginase activity of homogenates of all four tissues was produced by the addition of Mn^{++} ions. A further increase occurred when the tissues were activated at 50°. The activity was proportional to the quantity of tissue employed in all instances except in the case of the unactivated liver homogenate, the activity of which was measured in the absence of added activator. Little importance is attached to the determination of the activity of the homogenate in the absence of added $MnCl_2$ because of the different dilutions of the tissues employed and because of possible contamination with activating ions, especially in the case of the epidermis.

TABLE II

Typical Results for Mouse Liver, Kidney, Epidermis, and Squamous Cell Carcinoma

The results are expressed in micrograms of urea liberated per mg. of fresh weight of tissue under the standard assay conditions.

Treatment	Quantity of homogenate*	Tissue			
		Liver	Carcinoma	Epidermis	Kidney
	ml				
No $MnCl_2$, no incubation	0.1	280	3	2	6
	0.3	333	3	2	6
With $MnCl_2$, no incubation	0.1	1088	21	7	9
	0.3	1093	18	7	9
Incubated in $MnCl_2$	0.1	2190†	158†	43†	13‡
	0.3	2094	159	46	12

* 0.1 ml. of liver homogenate contained 0.05 mg. of fresh tissue, 0.1 ml. of the homogenates of the other tissues contained 1 mg. of fresh tissue.

† Incubated for 5 hrs. at 50°.

‡ Incubated for 1 hr. at 50°.

Reproducibility of Results—A 2 per cent homogenate was made from epidermis of normal mice and was divided into four aliquots. Each aliquot was studied in the manner previously described. At each level of activation the measurements on the separate aliquots were in excellent agreement with each other.

Summary of Assay Results for Liver, Kidney, Epidermis, and Carcinoma (Table III)—The liver has the greatest activity of the tissues studied with the carcinoma, epidermis, and kidney following in that order. The smallest degree of activation by the procedures employed was found in the kidney, the greatest activation was observed in the carcinoma.

Influence of Injection of Arginine on Arginase Activity of the Liver, Kidney,

and Epidermis (*Table IV*)—An experiment was performed in which 75 mg of arginine (pH 7 0) contained in 0 5 ml were injected daily into adult mice, while control mice were given the same quantity of physiological saline The mice were killed at the times indicated Only the values for the maxi

TABLE III

Arginase Activity of Mouse Liver, Kidney, Epidermis, and Squamous Cell Carcinoma

The results are expressed in micrograms of urea liberated per mg of fresh weight of tissue under the standard assay conditions All activities were measured in the presence of Mn^{++} ions

Sample No	Tissue							
	Liver		Carcinoma		Epidermis		Kidney	
	Before incubation	After incubation*	Before incubation	After incubation*	Before incubation	After incubation*	Before incubation	After incubation†
1	1179	2740	19	159	7	45	8	12
2	1090	2142	10	79	4	42	9	10
3	935	2330	29	220	5	31	13	16
4	1033	2490	22	206	5	21	9	9
5	1240	2565	24	191	2	22	3	6
Mean	1095	2453	21	171	5	32	8	11

* After incubation for 5 hrs at 50°

† After incubation for 1 hr at 50°

TABLE IV

Influence of Injection of Arginine on Arginase Activity of Liver, Kidney, and Epidermis

The results are expressed in micrograms of urea liberated per mg of fresh weight of tissue under the standard assay conditions Liver and epidermal homogenates were incubated with $MnCl_2$ for 5 hrs at 50°, kidney for 1 hr

Tissue*	Days after first injection	Control†	Arginine‡
Liver	6, 7, 10	2231 (1928–2740)	2013 (1587–2676)
Epidermis	6, 7, 8	40 (20–57)	40 (35–53)
Kidneys	6, 7, 10	10 (6–13)	12 (9–13)

* Four samples were studied for each tissue

† Received daily intraperitoneal injections of 0 5 ml of physiological saline

‡ Received daily intraperitoneal injections of 75 mg of arginine contained in 0 5 ml of a solution adjusted to pH 7 0

mally activated homogenates are reported It is apparent that the arginine injections produced no significant effect on the arginase activity of the tissues studied It has been reported that the injection of arginine markedly increased the arginase content of the muscles and kidneys of normal guinea pigs (5) The differing results for the kidneys may possibly be

ascribed to the different species employed or to variations in experimental procedures

DISCUSSION

The type of metallic activation that occurs in the case of arginase has also been described for leucine aminopeptidase (6, 7) and prolidase (8) It has been suggested that the activation of arginase consists in the transformation of a proarginase which contains no Mn into an active arginase containing Mn (3) However, the mechanism of the transformation is still not clear There is no way of estimating the actual activity of the enzyme in the active tissue prior to homogenization and dilution Therefore, little can be said regarding the physiological significance of determinations made in homogenates until these measurements are correlated with other variables to which they may be related

It was found that different degrees of activation could be achieved for the various tissues studied It is important to measure the maximal activity of which a tissue is capable, since this gives an idea of the potentialities of a tissue with respect to the reaction which the enzyme catalyzes It will be shown in a subsequent report on changes in arginase activity in epidermal carcinogenesis that the most consistent results and those best correlated with other chemical changes were obtained in the maximally activated tissues

From the results obtained it is apparent that any procedure employed for the measurement of the arginase activity of a tissue must be standardized for that tissue It would not even seem advisable to transfer a procedure developed for an organ of one species to the same organ of another species without further testing

SUMMARY

1 A procedure for the determination of the arginase activity of homogenates of tissue was developed from previously reported knowledge of the behavior of this enzyme and was applied to the estimation of the activity of the liver, kidney, and epidermis of the Swiss mouse and of a transplantable squamous cell carcinoma originally derived from the epidermis by the application of methylcholanthrene

2 The arginase activity of dilute homogenates of the above tissues was increased by the addition of Mn^{++} and was further increased by heating at 50° in the presence of Mn^{++}

3 The activity was proportional to the quantity of tissue employed and the rate of the reaction was constant under suitably chosen conditions

4 The liver had the greatest activity, with the carcinoma, epidermis, and kidney following in that order

5 The injection of arginine had no significant effect on arginase activity of liver, kidney, or epidermis in the mouse

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THE INACTIVATION OF STREPTOMYCIN BY CYANATE*

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The reversal of the bacteriostatic action of streptomycin for certain mycobacteria as well as *Escherichia coli* by urea has been the subject of a preliminary note by Fitzgerald and Bernheim (1) At that time the possibility was considered that urea was being directly assimilated by the organisms Subsequent work to elucidate the nature of this phenomenon has revealed that the agent which causes the reversal of streptomycin is most probably not urea but some product formed when urea is autoclaved in the medium The purpose of this paper is to present the experimental evidence for this conclusion as well as information on the possible nature of the active reverser and its mode of action

Methods

The experiments were done with *Mycobacterium tuberculosis* 607 grown in the Tween medium of Dubos (2) The medium was dispensed in a final volume of 10 cc in test-tubes 1 inch in diameter and suitable for use in the Evelyn photoelectric colorimeter The inoculum consisted of 0 5 cc of a 1 20 dilution of a 48 to 72 hour culture which was adjusted to a standard density prior to dilution All tests were run at 37° Ammonia determinations were done by a modified method of Van Slyke and Cullen (3) and urea determinations were done by the method of Ormsby (4) Stock 1 per cent solutions of urea were made in 0 05 M phosphate buffer at pH 7 0 and appropriate dilutions made in the medium prior to sterilization by autoclaving at 122° for 10 minutes Originally this procedure was followed routinely, since only about 10 to 20 per cent of the urea was decomposed by this treatment

The streptomycin was added aseptically from suitable dilutions of a stock solution of streptomycin sulfate (Winthrop) in sterile distilled water which contained 100,000 γ of streptomycin per ml Growth was followed turbidimetrically be means of an Evelyn colorimeter with a No 660 filter

EXPERIMENTAL

The effect of various concentrations of urea autoclaved in Dubos medium on the bacteriostatic action of 100 γ per cent of streptomycin is shown in

* Aided by a grant from the Duke University Research Council

Fig 1 Under the test conditions the growth of *Mycobacterium tuberculosis* 607 is inhibited by 10 0 γ per cent of the drug, while in the presence of 12 0 mg per cent of urea appreciable growth occurs with 100 0 γ per cent of streptomycin Subcultures from the urea-streptomycin tubes onto veal infusion-glycerin agar containing various concentrations of streptomycin revealed that the sensitivity of these organisms to the drug was the same as the controls In other words the organisms growing in the urea-streptomycin tubes had not become streptomycin-fast in spite of the fact that

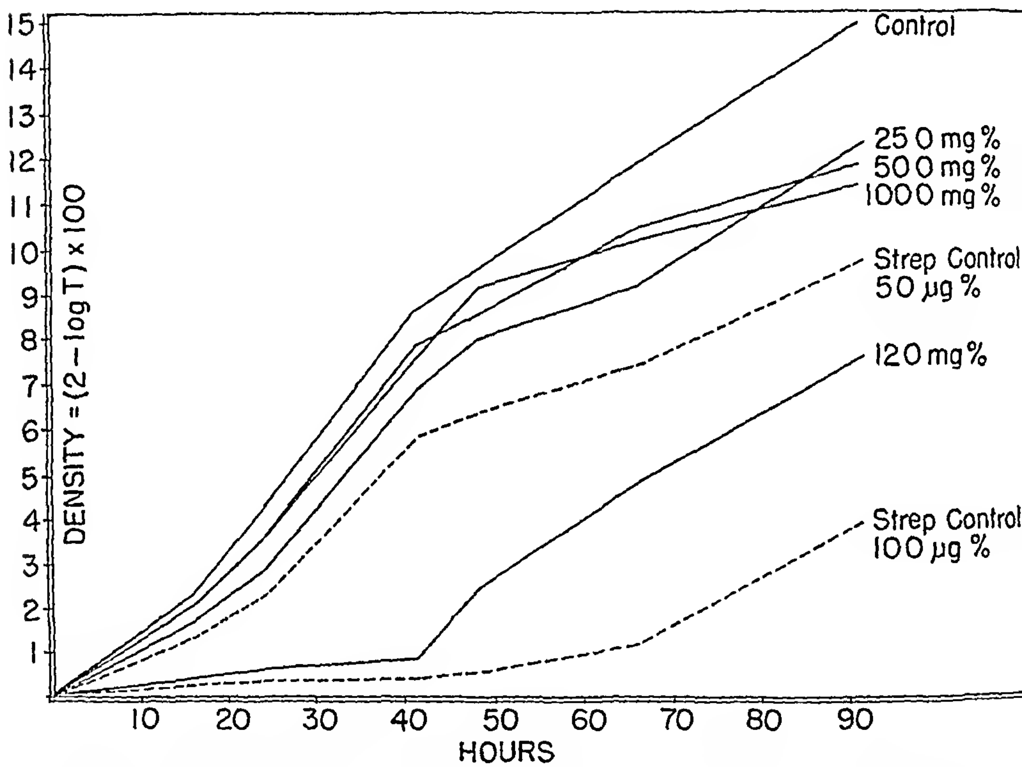

Fig 1 The effect of urea concentration on the bacteriostasis of *Mycobacterium tuberculosis* 607 by 100 0 γ per cent of streptomycin

under normal conditions *M tuberculosis* 607 can rapidly develop resistance to this drug The urea concentration of the medium decreases with growth of the organisms and, since similar results could be obtained with *Escherichia coli* 6522 which presumably contained no urease, the possibility was considered that urea was being assimilated directly by the organisms However, it was found that any loss of urea could be accounted for by accumulation of ammonia in the medium Subsequent studies showed that *M tuberculosis* 607, *M tuberculosis* BCG 8240, and the H37RV strain of *M tuberculosis*, as well as *E coli* 6522, all possessed definite urease activity

Furthermore it was found that no reversal of streptomycin occurred when the urea was sterilized by filtration through Seitz pads or sintered glass filters and added aseptically to the medium This would indicate that the active reverser is produced as a result of autoclaving the urea In order to determine the conditions necessary for the formation of the reverser, urea was autoclaved separately in distilled water and also together with various components of the medium prior to addition to the test medium The results of such an experiment are shown in Table I from which it may be seen that the most effective reversal of streptomycin occurs when the urea is autoclaved in the complete medium

TABLE I

Reversal of Streptomycin by Urea Previously Autoclaved With Different Constituents of Dubos Medium

Supplement*	Streptomycin	Density, (2 − log T) × 100		
		26 hrs	42 hrs	70 hrs
	γ per cent			
Urea and phosphates	100	0 0	0 0	2 4
	50	0 0	2 4	6.9
" citrates, and $MgSO_4 \cdot 7H_2O$	100	0 9	1 4	6 7
	50	2 4	6 7	8.3
Urea and complete medium	100	3 1	6 4	8 0
	50	3 4	7 3	8 6
" " distilled water	100	0 0	0 0	4 4
	50	0 0	0 0	0 0
Streptomycin control	100	0 0	0 0	0 0
	50	0 0	0 0	0 0
	5	0 0	0 9	7 4
Control	0	2 5	8 8	13 7

* Unless otherwise indicated, each tube contained the equivalent of 100 mg per cent of urea (before autoclaving) The various supplements as well as the complete medium were autoclaved for 10 minutes at 122°

To establish somewhat more precisely the conditions for the formation of the reverser samples of the urea-containing medium were boiled for 10 minutes or autoclaved for various periods of time Neither boiling for 10 minutes nor autoclaving for 1 minute gave rise to any reverser Best results were obtained when the urea-containing medium was autoclaved for from 5 to 15 minutes Growth in media with urea autoclaved for longer periods was not optimal, presumably due to the pH changes resulting from accumulation of ammonia

Removal of the residual urea after it had been autoclaved in the medium was accomplished by addition of purified urease (Squibb) It could be shown after such treatment that the reverser was still present

A number of compounds that could be considered related to or derived from urea were tested as possible reversers These included ammonium carbonate, ammonium carbamate, biuret, guanidine hydrochloride, thiourea, sodium cyanide, sodium thiocyanate, and potassium cyanate All the compounds were made up in 0 05 M phosphate buffer at pH 7 0 and sterilized by Seitz filtration Additions were made to the test mixtures from appropriate dilutions in Dubos medium None of these compounds could function as a reverser, with the exception of potassium cyanate

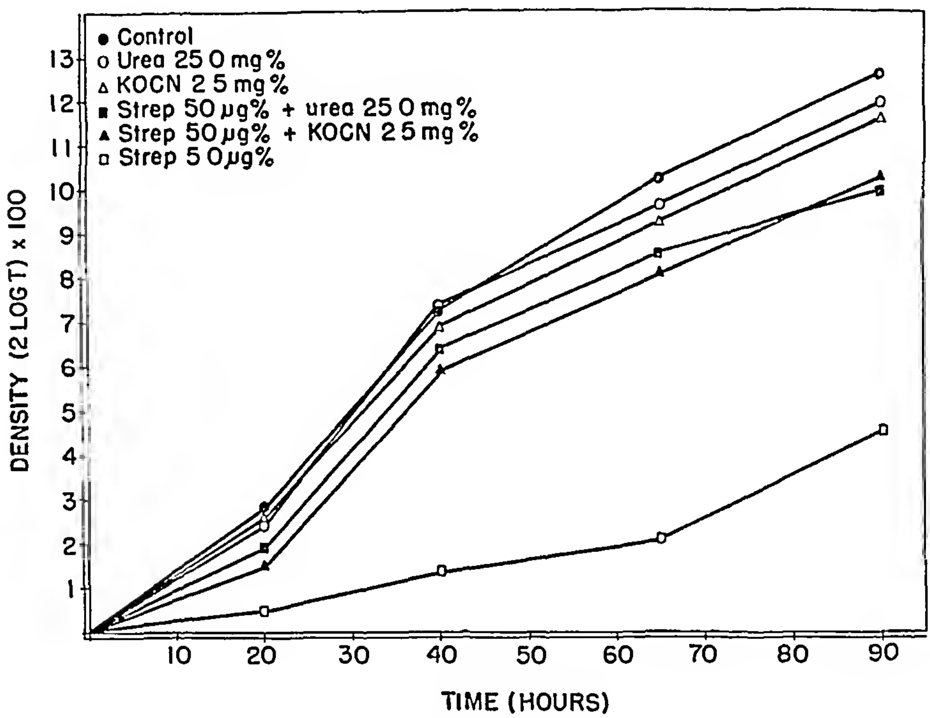

FIG 2 Comparison of urea autoclaved in the medium and potassium cyanate added aseptically as reversers of streptomycin

Fig 2 shows a comparison of potassium cyanate added aseptically and urea autoclaved in the medium as streptomycin reversers The cyanate has the greater activity, being approximately 10 times as effective as the autoclaved urea in counteracting the bacteriostatic effect of streptomycin on *Mycobacterium tuberculosis* 607 Similar results were obtained with *Escherichia coli* 6522 in the medium of Kohn and Harris (5), and a laboratory strain of *Staphylococcus aureus* in Difco nutrient broth When the cyanate was autoclaved in the medium most of its ability to reverse streptomycin was lost

Cyanate is probably not utilized by these organisms because concentrations above 10 mg per cent inhibit growth It seemed probable therefore

that cyanate was reacting with the streptomycin molecule If this were so, cyanate added to washed suspensions of mycobacteria should counteract the inhibitory effect of streptomycin on the formation of the adaptive enzyme for benzoic acid (6) Experiments were done with *Mycobacterium tuberculosis* 607 and *Mycobacterium lacticola* 1 0 mg of potassium cyanate completely prevented the inhibition of enzyme formation by 0 2 mg of streptomycin Since cyanate alone inhibits the oxygen uptake under these conditions it probably is not metabolized This experiment indicates therefore that cyanate reacts with the streptomycin molecule Because of the ability of cyanate to react with amino groups, it is probable that it combines with these groups in streptomycin

DISCUSSION

The inhibition of the bacteriostatic effect of streptomycin by urea autoclaved in Dubos medium is most probably due to formation of some new compound, since urea itself does not possess this property Salt effects on streptomycin are ruled out because comparable concentrations of ammonium carbonate or ammonium carbamate are inactive Of the compounds tested potassium cyanate is the only one which meets the requirements of the hypothetical compound in that it reverses streptomycin and small amounts of cyanates may be formed by autoclaving urea solutions

In view of the fact that cyanate does not seem to be a normal metabolite of *Mycobacterium tuberculosis* 607 and may actually inhibit its growth and metabolic activity it is probable that it reacts with the streptomycin molecule Although the nature of this reaction has not been elucidated, the reactivity of cyanates with free amino groups and the presence of these groups in the streptamine portion of the molecule suggest that this is the point of attack Donovick *et al* (7) have already demonstrated that streptomycin may be inactivated by certain carbonyl reagents such as semicarbazide, thiosemicarbazide, and hydroxylamine which presumably act upon the carbohydrate portion of the molecule It would appear from the results herein presented that the free amino groups of streptomycin are also essential for antibiotic activity

SUMMARY

1 When urea is autoclaved in Dubos medium a product is formed which blocks the bacteriostatic action of streptomycin

2 Of the substances tested cyanate is the only one which satisfies the characteristics of this reverser

3 Cyanate probably inactivates streptomycin directly, possibly by combining with its free amino groups

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THE REDUCTION OF COZYMASE BY SODIUM BOROHYDRIDE*

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Cozymase (diphosphopyridine nucleotide, DPN) has been assayed by a variety of biochemical and chemical methods (1) Among the simplest procedures has been the determination of the light absorption of reduced DPN at 340 mμ after reduction by sodium hydrosulfite Since special precautions are required to remove completely the excess reducing agent which absorbs in this region, it appeared advantageous to find another reducing agent which could be employed for the same purpose with greater ease

A study of the action of sodium borohydride ($NaBH_4$) on DPN revealed that it filled the necessary requirements The main advantage of this new reagent lies in its absence of light absorption at 340 mμ Spectrophotometric measurement of DPN reduced by $NaBH_4$ may therefore be made without removal of excess reagent The assay procedure together with some observations on the properties of the reduction product will be described in this communication

EXPERIMENTAL

Reagents—Sodium borohydride was obtained as a white powder of approximately 95 per cent purity [1] Its preparation and some of its properties have been previously described (2) In water solution, $NaBH_4$ hydrolyzes slowly according to the equation $NaBH_4 + 2H_2O \rightarrow Na^+ + BO_2^- + 4H_2$

This reagent was selected from a variety of available metallo hydrides because it shows a moderate stability in water solution The rate of hydrolysis was determined approximately by titrating $NaBH_4$ with iodine, and it was ascertained that a 0 1 per cent solution in distilled water was completely hydrolyzed in 1 minute at 100°, but that at 4° only about 10 per cent of the available reducing ability of a 5 per cent solution was lost per day These properties make it possible to destroy excess reagent completely when this is necessary, and also to prepare the reagent for use as a solution which may be kept in the cold for several hours

Cozymase was purchased from the Schwarz Laboratories Solid dihydrocozymase was prepared according to Ohlmeyer (3) The assay of DPN

* This investigation was aided by a grant from the Rockefeller Foundation

[1] Kindly furnished by Albert Stewart, Chemistry Department, University of Chicago

with sodium hydrosulfite was carried out according to the procedure used by LePage (4).

All spectrophotometric measurements were made with a Beckman quartz spectrophotometer, model DU.

Assay of DPN with $NaBH_4$—The assay of DPN is accomplished by adding about 2 mg. of $NaBH_4$ (as solid or in solution) to 3.0 cc. of 0.1 M phosphate buffer, pH 7.0, containing about 0.5 mg. of DPN. These conditions are suitable for cells of 1 cm. width commonly used with the Beckman spectrophotometer. About 1 minute is allowed for the reduction. During this time the solution is shaken gently to aid removal of hydrogen formed by hydrolysis of the reagent. The optical density is then measured at once. Interference with the measurement by gas bubbles can readily be avoided by proper tilting of the cuvette immediately before the reading is made. Phosphate buffer may be used as a blank, but corrections must of course be made for any light absorption by impurities in the unreduced DPN preparation.[2]

TABLE I

Assay of Cozymase Preparations

	Per cent purity*	
	Lot 1	Lot 2
By reduction with sodium hydrosulfite (LePage (4))	37.6 ± 0.4	40.5 ± 1.0
" " " " borohydride, spectrophotometric	38.1 ± 0.2	39.9 ± 0.8
By reduction with sodium borohydride, iodine titration	39.1 ± 0.5	41.6 ± 0.5

* Data are given with the average deviation from the mean of two or more determinations.

The validity of the method rests upon the fact that the spectrum of the reduction product is identical with that obtained with sodium hydrosulfite for the region 330 to 400 mμ. The absorption band with a peak at 265 mμ, characteristic also of adenine derivatives present as impurities in the DPN preparations used, was not appreciably changed by $NaBH_4$. Table I

[2] It is necessary to use a buffer of pH 7.0, since the reduction of DPN by $NaBH_4$ in solutions of pH greater than 7.5 leads to the production of a stable yellow color possessing an absorption peak at 340 mμ and a smaller peak at about 420 mμ. This does not occur at pH 7.0 to 7.1. A stable yellow product has been obtained by Adler and coworkers (5) on reduction of DPN with sodium hydrosulfite in highly alkaline medium. It has commonly been supposed to be a semiquinoid radical (6). The stable yellow substance obtained upon reduction of DPN with $NaBH_4$ in media of pH greater than 7.5 differs considerably from the above product in absorption spectrum and will be reported on more fully in a subsequent communication.

shows representative results obtained on assaying two different DPN preparations spectrophotometrically, by means of both the $NaBH_4$ and the hydrosulfite procedures The percentage purity was calculated from an extinction coefficient of 6 27 × 10^6 sq cm per mole at 340 mμ (3)

Table I also shows the assay values obtained by iodine titration of the reduced DPN formed by $NaBH_4$ Titration of reduced DPN was carried out with 0 005 N iodine solution and starch as indicator This method was first suggested by Karrer and Ringier (7) and has more recently been used by Drabkin (8) as a method for assaying solid dihydrocozymase 1 mole of pure dihydrocozymase is quantitatively oxidized by 2 equivalents of iodine For the determinations reported in Table I, the reduction was carried out as described for the spectrophotometric assay with a somewhat larger amount of the reactants, and excess $NaBH_4$ was then removed by heating to 100° for 1 minute

Biological Activity of Product—The enzymatic reoxidation of reduced DPN was carried out with sodium pyruvate and lactic dehydrogenase obtained by dialysis of an extract of a pig heart acetone powder In no case did enzyme alone or substrate alone produce any reoxidation Reduced DPN obtained by use of sodium hydrosulfite was found to be 100 per cent reoxidizable by lactic dehydrogenase, as was demonstrated by Green and Dewan (9) Under similar conditions, DPN reduced by $NaBH_4$ was reoxidized only 45 to 51 per cent Partial enzymatic reoxidation of $NaBH_4$-reduced DPN lowered the absorption band with a peak at 340 mμ in the same proportion at each wave-length over the range of 330 to 400 mμ

It has been earlier observed that sodium bisulfite and hydrogen cyanide combine with DPN (but not with reduced DPN) to yield addition products absorbing at 340 mμ (10) That there was no interference from comparable addition products in the reoxidation of DPN reduced by $NaBH_4$ follows from the unchanged optical density obtained on the addition of a completely hydrolyzed solution of $NaBH_4$ to DPN

DISCUSSION

The agreement between assay values appearing in Table I shows that sodium borohydride may be satisfactorily used for the assay of DPN [3] The products of reduction, however, though closely similar, are not entirely identical to those obtained by the use of sodium hydrosulfite

The probability that reduction by sodium borohydride produced two isomeric forms of dihydrocozymase should be considered Since, as

[3] In applying $NaBH_4$ to the analysis of triphosphopyridine nucleotide, Mr Eric Conn of the Department of Biochemistry, University of Chicago, has found that the results are in agreement with those obtained by the reduction of triphosphopyridine nucleotide with glucose 6 phosphate and *Zwischenferment*

pointed out by Karrer and coworkers (11), the two possible isomeric dihydro products would be expected to differ in ultraviolet spectrum, it seems more probable that sodium borohydride acts upon DPN in a more complex manner

SUMMARY

Sodium borohydride has been applied successfully to the assay of DPN Since this reagent does not absorb in the near ultraviolet and the excess is readily hydrolyzed to sodium borate, the new assay procedures are more convenient than previous ones with sodium hydrosulfite However, the reduced products obtained are only partially enzymatically active

I should like to express my thanks to Professor Birgit Vennesland and to Professor T R Hogness for their kind criticism and encouragement during the course of this work

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CARBOHYDRATE METABOLISM IN HIGHER PLANTS

I PEA ALDOLASE

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In recent years muscle aldolase has been studied extensively by several investigators (1–5) The enzyme has been crystallized and its properties and kinetics carefully documented Little attention, however, has been paid to its counterpart in plant tissue Although its presence in plants has been indicated by several workers, namely Baba (6), Allen (7), and James *et al* (8), its properties, kinetics, and biological function have not as yet been analyzed

The purpose of this communication is to present such data for a plant aldolase isolated from the pea seed Subsequent communications will deal with the distribution and biological rôle of aldolase in higher plants and other key enzymes involved in carbohydrate metabolism in higher plants

Preparation and Properties

For the most part pea seeds (Dwarf Telephone) were employed as the source for the enzyme, though squash seeds were also found suitable The enzyme was readily extracted from pea seeds, which had been soaked for 12 hours in distilled water at 2°, with 0 1 per cent potassium carbonate as the extracting solvent The extract was then subjected to (1) ammonium sulfate fractionation, (2) isoelectric precipitation with dilute acetic acid at pH 5 5, by which procedure much inert protein was precipitated, while aldolase remained in solution, and finally (3) acetone fractionation followed by dialysis and a final isoelectric precipitation The details of the purification procedure are summarized in Table I and discussed in the experimental section

Enzyme activity was measured by the cyanide-fixing method of Herbert *et al* (1), in which the triose phosphates, formed from the scission of fructose diphosphate, react with cyanide to yield cyanohydrins Triose phosphate P was then estimated after hydrolysis by exposure to strong alkali for 20 minutes at 20° The enzyme unit employed in this investigation differed from others, since pea aldolase does not have the high specific activity of its counterpart in animal tissue Therefore, in establishing a reproducible unit, the amount of alkali-labile P released has been decreased 10-fold, while the time period was increased from 3 to 10 minutes The unit may then be defined as the amount of enzyme required to liberate

0 1 mg of inorganic P equivalent to triose phosphate in 10 minutes at 30° in a 0 1 M veronal buffer of pH 8 5 in an excess of fructose diphosphate and cyanide Other carbonyl fixatives, such as bisulfite, hydrazine, and semicarbazide, in final concentrations equivalent to that of cyanide (0 08 M) can be employed Hydroxylamine was unsatisfactory because it inhibited aldolase completely in a 0 01 M final concentration

The final enzyme fraction of highest purity (Fraction Bs) is a water-clear solution with no isomerase activity Since the initial extract contained 0 06 unit per mg of protein and the final preparation 5 5 units per mg of

TABLE I

Purification of Pea Aldolase

Fraction	Purification procedure	Total units	Units per mg protein
Extract		460	0 06
I	Neutral saturated $(NH_4)_2SO_4$ to 35% saturation	43	0 007
II	" " " " 70% "	341	0 11
	II dialyzed 5 hrs against distilled water at 50°		
IIa	Neutral saturated $(NH_4)_2SO_4$ to 40% saturation	14	0 03
IIb	" " " " 51% "	98	0 14
IIc	" " " " 61% "	165	0 19
IId	" " " " 70% "	34	0 07
	IIc dialyzed overnight against distilled water		
IIc, residue	Ppt with 1% acetic acid to pH 5 5	6	0 04
" supernatant (A)		155	0 25
A	Acetone added to 55%	5	0 08
B	" " " 62%	135	0 32
C	" " " 72%	2	
	B dialyzed overnight		
B, residue	Ppt with 1% acetic acid to pH 5 5	8	0 11
" supernatant (Fraction Bs)		97	5 50

protein, the degree of purification relative to the first extract is 5 5/0 06 or 92-fold 1 mg of protein (Fraction Bs) forms 3 3 mg of triose phosphate P per hour or 18 1 mg of triose phosphate per hour under standard conditions at 30°

The enzyme is strongly adsorbed on alumina C γ All attempts to elute with dilute alkaline solutions, including veronal buffers, sodium bicarbonate, and ammoniacal ammonium sulfate, failed Some attempts were made to induce crystallization by the techniques described recently by Taylor *et al* (3), but without success

The enzyme is a rather stable protein Solutions of the enzyme can be

frozen and stored indefinitely without loss of activity Dialysis in the cold against distilled water resulted in no loss, though dialysis at room temperature did cause appreciable loss It is unstable below pH 5 5 and above 10, but stable within these ranges Organic solvents, such as acetone or alcohol, did not alter its stability

Exposure of a neutral solution of the enzyme for 5 minutes at 45° caused no inactivation However, at 50° a 4 per cent loss was observed, at 55° a 70 per cent loss, and at 60° complete inactivation occurred

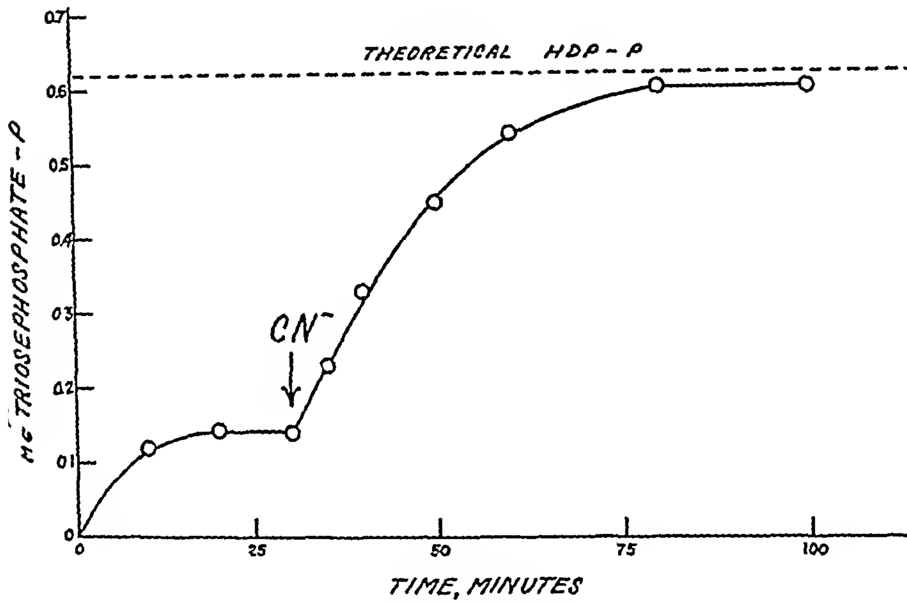

FIG 1 Effect of time on decomposition of hexose diphosphate Each tube contained 1 0 cc of enzyme (Fraction Bs), 0 5 cc of 0 1 M veronal buffer at pH 8 5, and 0 1 cc of 0 1 M hexose diphosphate At a given time 0 1 cc of 2 M cyanide at pH 8 5 was added Temperature 31°

Reaction—Pea aldolase catalyzes the reversible reaction

(1) Fructose diphosphate ⇄ dihydroxyacetone phosphate + D-3 glyceraldehyde phosphate

The reversible nature of this reaction is illustrated in Fig 1 Equilibrium was attained in approximately 10 minutes Addition of cyanide to the reaction mixture fixed the triose phosphates and thus the reaction was shifted completely to the right Within 70 minutes hexose diphosphate was quantitatively converted to an equimolar mixture of dihydroxyacetone phosphate and D-3-glyceraldehyde phosphate

Iodine oxidation experiments with equilibrium mixtures demonstrated that equimolar mixtures of the triose phosphates were formed Therefore it may be concluded that isomerase was absent from the enzyme preparations employed in equilibrium studies Although both triose phosphates are unstable in strong alkali, iodine oxidation converts D-3-glyceraldehyde phosphate, but not dihydroxyacetone phosphate, to the very stable phosphoglyceric acid Thus, after iodine oxidation of an equilibrium mixture, if no isomerase is present, the alkali-labile phosphate, derived now solely from the degradation of dihydroxyacetone phosphate, should fall to 50 per cent or less of the original alkali-labile phosphate Experimentally, after iodine oxidation of different equilibrium mixtures, a decrease of from 50 to 70 per cent of the original total alkali-labile phosphate was consistently observed

The equilibrium constant K was therefore estimated from the equation

$$K = \frac{(\tfrac{1}{2}\text{ triose phosphate})^2}{(\text{fructose diphosphate})} \tag{2}$$

and was found to be about $1\ 15 \times 10^{-4}$ mole per liter at 31° (in borate buffer at pH 8 5), which is somewhat higher than that of animal aldolase which is about $0\ 9 \times 10^{-4}$ mole per liter at 31° (1, 2)[1] As summarized in Table II, the equilibrium is shifted to the right with an increase in temperature and therefore follows the van't Hoff law of reaction isochores

Pea aldolase like animal aldolase exhibits the same high substrate specificity in that only fructose-1,6-diphosphate is broken down to the triose phosphates The monophosphates of glucose, glucose-1-phosphate and glucose-6-phosphate, and the monophosphate of fructose, fructose-6-phosphate, are inert in the enzyme system

The pH optimum, shown in Fig 2, is approximately 8 5 in 0 1 M veronal buffer Since the enzyme protein is unstable in the higher as well as in the lower pH ranges (above pH 10 and below pH 5), the fall in activity is due to a destruction of the enzyme protein rather than to a reversible decrease in enzyme activity In setting up the pH dependence curve, all pH adjustments of reaction mixtures were controlled with the Beckman pH meter

Figs 3 and 4 indicate the relation of enzyme activity to enzyme concen-

[1] The slight discrepancy between the observed K values for pea aldolase and the values cited for the animal aldolase could not be reconciled despite the following considerations (a) since isomerase is absent, the differences observed could not be caused by K measurements of two reactions, catalyzed by aldolase and isomerase, (b) phosphatase is absent since no inorganic P is detected in reaction mixtures, (c) though borate buffers at pH 8 5 were employed, buffers of pH 7 3 used by Meyerhof and Herbert gave similar results, (d) experiments with muscle aldolase gave K values which checked closely with values in the literature

TABLE II

Aldolase Equilibrium at Different Temperatures*

Enzyme	Temperature	Time	Hexose diphosphate final	Triose phosphate formed	K	K (mean)
cc	C	min	M × 10⁻³	M × 10⁻³	M × 10⁻⁴ per l	M × 10⁻⁴ per l
0 5	31	20	3 83	1 40	1 28	
0 5		20	8 03	2 06	1 32	
0 5		40	8 01	2 1	1 37	
0 5		30	6 27	1 42	0 80	
0 5		20	4 07	1 46	1 31	
1 0		20	4 05	1 50	1 39	
0 5		30	3 9	1 2	0 92	1 15
0 5	22	40	3 94	1 18	0 88	
1 0		40	3 92	1 22	0 95	0 91
1 0	40	20	3 23	2 6	5 25	
1 0		40	7 03	4 07	5 87	5 56

* In 0 1 M borate buffer at pH 8 5

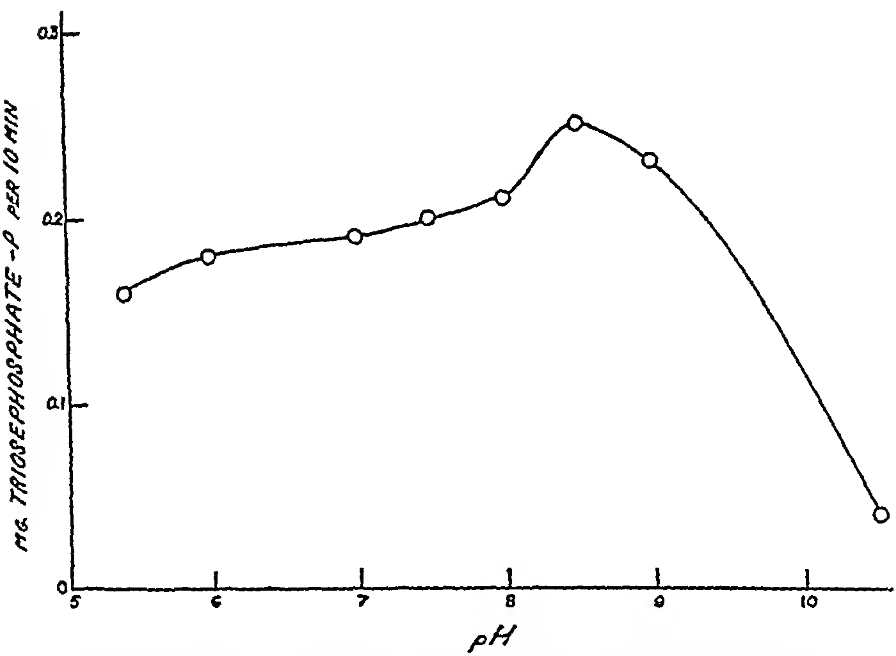

FIG 2 Reaction velocity as a function of pH Each tube contained 1 cc of enzyme, 0 5 cc of 0 1 M hexose diphosphate, 1 cc of 0 1 M veronal buffer, and 1 cc of 0 25 M cyanide, the reaction mixture being adjusted to the desired pH 0 1 M acetate buffer was employed for the value at pH 5 4 Temperature 31°

tration and substrate concentration, respectively From Fig 4, the K_m is approximately $0\,8 \times 10^{-3}$ mole per liter, which suggests that pea aldolase has an affinity for the substrate some 10-fold greater than that of animal aldolase, which has a K_m of about 9×10^{-3} mole

In sharp contrast to the animal enzyme (1, 4), pea aldolase is not very sensitive to heavy metal inhibition Thus in a final concentration of 10^{-4} M, copper sulfate, mercuric acetate, phenylmercuric acetate, and silver nitrate did not inhibit the enzyme Further, in a final concentration of

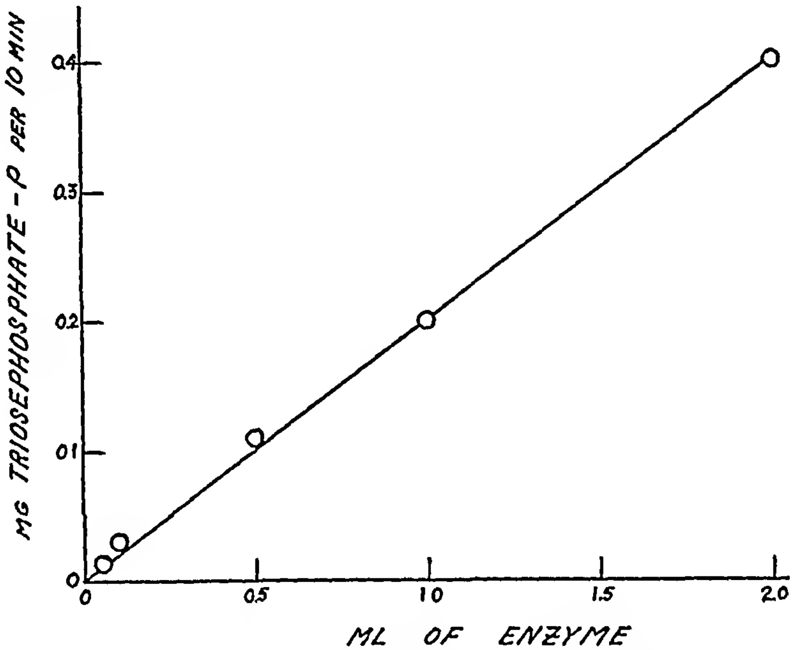

FIG 3 Reaction velocity as a function of enzyme concentration Each tube contained 0 5 cc of 0 1 M hexose diphosphate, 1 cc of 0 1 M veronal buffer at pH 8 5, 1 cc of 0 25 M cyanide at pH 8 5, varying concentrations of enzyme, and water to a final volume of 4 5 cc Temperature 31°

10^{-3} M iodoacetamide, fluoride, indoleacetate, and azide as well as a saturated solution of capryl alcohol and 10^{-4} N iodine gave no inhibition Cysteine, α,α'-dipyridyl, and cyanide, contrary to the observations of Warburg and Christian with yeast aldolase (5), did not inhibit the enzyme

Several compounds which might be considered as possible competitive inhibitors for the active center of the enzyme were examined In final concentrations of 10^{-2} M, glucose, fructose, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, β-glycerophosphate, and pyruvic acid did not cause any decrease in the rate of fructose diphosphate decomposition

Of the several carbonyl reagents tested, cyanide, bisulfite, semicarbazide, hydrazine (adjusted to a final pH of 8 5) in a final concentration of 8×10^{-2}

M were equivalent in their fixative capacity Hydroxylamine, however, was the only exception, since it acted as an inhibitor of the enzyme Thus in a final concentration of 10^{-2} M, the reagent inhibited the enzyme 100 per cent, in 10^{-3} M, 55 per cent, and in 10^{-4} M, only 14 per cent Since divalent cations such as cobalt, zinc, magnesium, and manganese did not reverse the inhibition, the nature of the inhibition was probably not related to the formation of a metal-reagent-enzyme complex

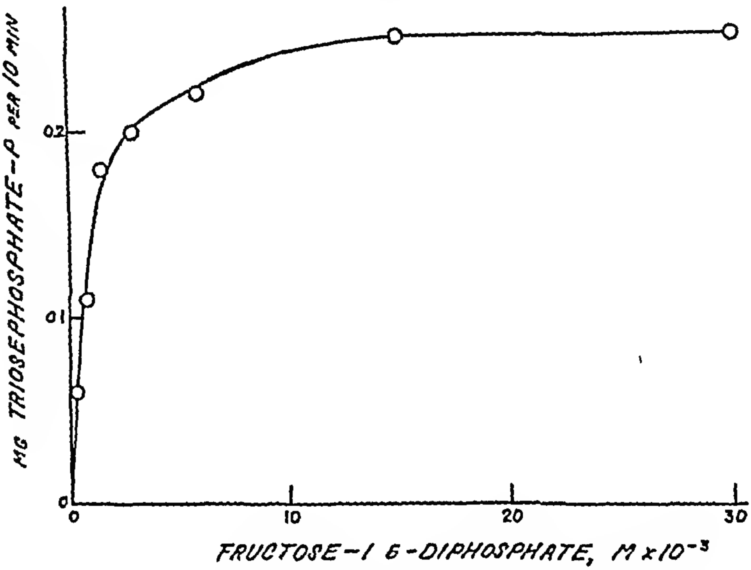

FIG 4 Reaction velocity as a function of substrate concentration Each tube contained 0 5 cc of enzyme, 1 cc of 0 25 M cyanide, 1 cc of 0 1 M veronal buffer at pH 8 5, increasing concentrations of 0 1 M hexose diphosphate, and water to a final volume of 3 5 cc Temperature 31°

Attempts to resolve the enzyme into a protein moiety and a specific prosthetic group were unsuccessful After dialysis for 12 hours at 2° against 10^{-2} M acetate buffer at pH 4 5, the enzyme was completely inactive, addition of either zinc, magnesium, manganese, or cobaltous ions to the dialyzed enzyme did not restore activity After dialysis for the same period of time against 0 05 per cent potassium carbonate, no loss in activity was observed Prolonged dialysis against distilled water and exposure of the enzyme to saturated ammonium sulfate at pH 4 5 also did not yield successful results

EXPERIMENTAL

Preparation—In a typical purification procedure, 25 gm of pea seeds (Dwarf Telephone), after soaking overnight at 2° in an equal volume of

distilled water, were suspended in 10 volumes of cold 0 1 per cent potassium carbonate and dispersed in a Waring blendor for 5 minutes The larger particles were removed by passing the extract through several layers of moist cheese-cloth Purification procedures were then employed, as outlined in Table I

In ammonium sulfate fractionation, neutralized saturated ammonium sulfate rather than unneutralized or solid ammonium sulfate was employed and found to give higher yields Since long periods of gravity filtration of ammonium sulfate precipitation at room temperature resulted in some loss of enzyme, all separations were therefore carried out in the cold by centrifuging in the International refrigerated model No 1 Rapid isoelectric precipitations with 1 per cent acetic acid at 0° to pH 5 5 were essential, since even short exposure to pH lower than 5 5 led to irreversible inactivation

All attempts to carry purification beyond the stage of 5 5 units per mg of protein were discontinued, since it was consistently observed that addition of either saturated ammonium sulfate or acetone to the 5 5 unit stage did not give clear cut fractions, instead stable colloidal solutions formed, from which proteins could be separated only after the removal of the added reagents by dialysis, etc

Methods of Estimation—Enzyme activity was estimated as follows 1 cc of an enzyme solution, 1 cc of 0 1 M veronal buffer at pH 8 5, and 1 cc of 0 25 M cyanide[2] in a test-tube were placed in a water bath maintained at 30° for 10 minutes 0 5 cc of 0 1 M fructose diphosphate (also at 30°) was rapidly added and the reaction stopped after 10 minutes by the addition of 5 cc of 10 per cent trichloroacetic acid The mixture was then diluted to 10 cc, filtered, and 0 5 cc aliquots were made strongly alkaline by adding 0 5 cc of 2 N NaOH After 10 minutes at room temperature, the alkaline mixture was neutralized and estimated for inorganic phosphate by the Fiske and Subbarow method (9) 0 5 cc aliquots, which were not exposed to alkali, were run simultaneously to correct for inorganic P blanks

Iodine oxidations were conducted as follows To a 0 5 cc trichloroacetate aliquot were added 1 5 cc of 10 per cent solution of sodium bicarbonate and 0 05 cc of 1 N iodine, this was allowed to react for 10 minutes at room temperature 0 1 cc of N bisulfite was then added to remove excess iodine and the alkali-labile phosphate was estimated as described above

Dry weights were determined by adding to tared 15 cc Pyrex centrifuge tubes known volumes of enzyme preparations Protein was precipitated by adding 5 cc of 10 per cent trichloroacetic acid, the mixture was centrifuged and washed two times with 10 cc of 1 per cent trichloroacetic acid

[2] At the beginning of each experiment, 0 25 M cyanide was freshly adjusted to pH 8 5 by adding 6 N HCl with phenolphthalein as the internal indicator

The precipitates in the centrifuge tubes were then dried at 80° for 12 hours and weighed Duplicate runs were carried out in each case

Substrates—Commercially available hexose diphosphates contained about 15 per cent inorganic phosphate (of the total phosphate) For routine experiments, hexose diphosphate was prepared by treating sodium hexose diphosphate with magnesia mixture Such preparations were free of inorganic phosphate and contained 84 per cent hexose diphosphate as determined enzymatically Purer samples of the substrate were obtained for equilibrium and resolution experiments by preparing the acid barium salt by the procedure of Neuberg *et al* (10) Such preparations were free of inorganic phosphate and were found to contain 98 per cent hexose diphosphate by aldolase analysis Barium fructose-6-phosphate was prepared by the method of Neuberg *et al* (10), potassium glucose-1-phosphate by a modified method of Sumner and Somers (11) We are indebted to Dr W Z Hassid for a generous sample of synthetic potassium glucose-6-phosphate

SUMMARY

An aldolase has been isolated from peas and has been purified some 92 times Its properties, kinetics, and equilibrium constant have been studied and compared with muscle aldolase Effects of inhibitors have also been investigated

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THE EFFECT OF CRYSTALLINE ADRENAL CORTICAL STEROIDS, DL-THYROXINE, AND EPINEPHRINE ON THE ALKALINE AND ACID PHOSPHATASES AND ARGINASE OF THE LIVER AND KIDNEY OF THE NORMAL ADULT RAT*

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Aqueous adrenal cortical extract (Upjohn), but not desoxycorticosterone acetate, produces a remarkable increase in the "alkaline" phosphatase of the liver of the adrenalectomized rat (1–3) This effect is apparently related to glyconeogenesis from endogenous protein (4) The extract, however, contains in addition to the C_{11} steroids many other substances including epinephrine[1] (less than 1 800,000), which is able to increase liver glycogen with lactic acid as a precursor It became important, therefore, to determine whether this enzyme phenomenon was a specific property of C_{11} adrenal cortical steroids and also to obtain information as to in what phase in the glyconeogenic process this enzyme was involved As a means to these ends, a comparison has been made of the effect of aqueous adrenal cortical extract with a crystalline mixture of hog adrenal cortical steroids (Upjohn's lipoextract),[2] 11-dehydrocorticosterone acetate (Merck, synthetic),[3] and epinephrine Furthermore, a study with thyroxine has been included because it is known to accelerate glycogenolysis due to an increase in energy demands

At the same time the arginase activity of the liver was studied in order to obtain further information as to the apparent discrepancy between the increase observed by Fraenkel-Conrat *et al* (5) and the lack of increase noted in this laboratory (4) after administration of adrenal cortical extracts to adrenalectomized rats

Procedure

Male rats of the Sprague-Dawley strain were placed in individual cages in an air-conditioned room at 25 5–26 6° and fed 10 gm per day of a pre-

* This investigation was aided by grants from the Josiah Macy, Jr , Foundation

[1] Personal communication, Dr D J Ingle, The Upjohn Company

[2] The lipoextract (Research No 8120) was provided by Dr M H Kuizenga of The Upjohn Company on June 21, 1943, and was kept at room temperature The experiments in this paper were carried out on February 16, 1948

[3] The synthetic 11 dehydrocorticosterone acetate was provided by Merck and Company on December 19, 1947, and dissolved at 5 mg per ml in sesame oil containing 10 per cent benzyl alcohol

paired diet composed of casein 16 7, sucrose 61 2, hydrogenated vegetable oil 7 4, yeast (Fleischmann's 2019) 9 2, Cellu flour 1 8, Wesson's salt mixture 3 7 (6), and as a daily supplement 1 drop of cod liver oil and 1 drop of a 34 per cent tocopherol concentrate of wheat germ oil[4] diluted 10-fold with Wesson oil The rats were kept 3 to 4 weeks on this regimen before the experiments were carried out

The adrenal cortical hormones were administered according to the procedure of Reinecke and Kendall (7) The epinephrine was injected 1 hour before autopsy as a single 0 05 ml subcutaneous dose of a 1 1000 commercial preparation of the hydrochloride (Parke, Davis and Company) The DL-thyroxine (Roche-Organon, synthetic) was dissolved in a small amount of 0 02 N sodium hydroxide and then made to 2 5 mg per ml with water The rats lost weight rapidly with the initial dose during the first 3 days, therefore, the amount was reduced to 0 25 mg per day for the remaining 2 days The last injection was made 24 hours before autopsy

At the end of the experiments the animals were anesthetized by the intraperitoneal injection of 0 3 ml of dial-urethane,[5] and the liver was removed and rapidly weighed on a Roller Smith torsion balance The left segment of the median lobe was used for the enzyme studies (2, 4) The remainder was placed immediately into 5 ml of hot 30 per cent potassium hydroxide and analyzed for glycogen by the Good-Kramer-Somogyi technique (8), except that the hydrolyzed glycogen was neutralized to phenolphthalein (9) The recently modified Somogyi method (10) was used to determine the reducing substance and the results are expressed as glucose

The kidneys were removed, weighed, and the left one used for the enzyme studies

The nitrogen content of the organs of the rats treated with DL-thyroxine and epinephrine was determined by the micro-Kjeldahl technique on aliquots of the enzyme homogenates

Results

Adrenal Cortical Hormone (*Table I*)—There was the expected formation of liver glycogen under the stimulation of these hormone preparations The most effective material in the doses used was the lipoextract, then the aqueous extract, and finally the synthetic 11-dehydrocorticosterone acetate The "alkaline" phosphatase of the liver was greatly increased and to approximately the same extent for each adrenal cortical preparation There was no parallelism with the degree of glyconeogenesis

The arginase activity and also the "acid" phosphatase of the liver were not significantly altered

[4] The wheat germ oil concentrate was provided by Distillation Products, Inc , through the courtesy of Dr P L Harris

[5] The dial-urethane was provided by Ciba Pharmaceutical Products, Inc

Epinephrine (*Table II*)—The injection of epinephrine produced within 1 hour a tremendous deposition of glycogen in the liver of rats fasted for 18

TABLE I

Effect of C_{11} Adrenal Cortical Steroids on Composition and Enzymes of Livers of Male Rats (250 to 252 gm)

Each group consisted of four rats

	Dose	Liver	Glycogen total	"Alkaline" phosphatase*	Arginase
		mg	*mg*	*units per gm per cent*†	*units per gm per cent*†
Control		6640	22	(3 3)	(11,590)
Adrenal cortical extract‡	8 × 1 ml per hr	7440	118	+164	−24
Lipoextract§	8 × 0 5 ml per hr	7060	154	+161	−17
11 Dehydrocorticosterone acetate (synthetic)‖	8 × 1 mg per hr	6270	46	+171	−11

* No effect on "acid" phosphatase of liver or enzymes of kidney
† Per cent change from control values which are given in parentheses
‡ Aqueous, Upjohn
§ Lipoextract = Upjohn's adrenal cortical extract 1 ml is equivalent to 2 mg of 11-dehydro-17 hydroxycorticosterone
‖ Dissolved at 5 mg per ml in sesame oil containing 10 per cent benzyl alcohol

TABLE II

Effect of Epinephrine and Thyroxine on Composition and Enzymes of Livers of Male Rats

Each group consisted of five rats

	Dose	Body weight	Liver	Glycogen total	Nitrogen total		"Alkaline" phosphatase	Arginase
		gm	*mg*	*mg*	*mg*	*per cent*	*units per gm per cent**	*units per gm per cent**
Control		287	8230	120	298	3 63	(3 0)	(11,310)
Epinephrine	0 05 mg †	286	9510	613	279	2 95	−5	−24
DL-Thyroxine	3 × 0 5 mg per day 2 × 0 25 mg per day	288‡	7610	7	284	3 71	−33	+3

* Per cent change from controls which are given in parentheses
† The autopsy was performed 1 hour after injection
‡ The body weight was 278 gm at autopsy

hours, which was accompanied by a proportionate decrease in the per cent but not total nitrogen (protein) content

The arginase and "alkaline" phosphatase were not significantly altered

DL-*Thyroxine* (*Table II*)—The animals injected with thyroxine lost 10 gm of their body weight with a slight but not significant increase in urinary nitrogen The liver glycogen was extremely low but the nitrogen (protein) content was comparable with that of the control rats

The "alkaline" phosphatase was somewhat decreased, but the arginase was not affected

The weight of the kidney as was expected (*cf* (11)) increased 22 per cent Part of this increase was due probably to fat deposition (12) The kidneys were putty-colored It is of interest that the kidneys increased in size while the rats were on a constant food intake In previous observations (11) the rats were fed *ad libitum*

In all of the above experiments there was no change in the "acid" phosphatase activity of the liver or any of the enzymes of the kidney

DISCUSSION

The similar increases in "alkaline" phosphatase of the liver of the rat after administration of aqueous adrenal cortical extract,[6] lipoextract, and synthetic 11-dehydrocorticosterone acetate indicate that this phenomenon is a property of the S hormones of the adrenal cortex

The inability of either epinephrine or thyroxine to influence the level of activity of this enzyme in the liver in spite of their marked glycogenic and glycogenolytic properties provides indirect evidence that the enzyme may be concerned with the endogenous protein or amino acid phase of glyconeogenesis This hypothesis gains further support from the fact that neither a high protein nor a high carbohydrate diet will produce comparable increases in the "alkaline" phosphatase of the liver (3)[7]

The inability of any of the C_{11} steroid preparations to change significantly the arginase level of the liver of the normal rat supports the results obtained in the adrenalectomized rat (3, 4) These data, therefore, provide further evidence that a change in liver arginase level is not essential for glyconeogenesis from protein under the stimulation of the C_{11} steroids as suggested by Fraenkel-Conrat *et al* (5, 13) Therefore, the decrease in the level of this enzyme after adrenalectomy (5, 4, 14) and hypophysectomy (13)[7] must be due to some other factor or group of factors (3, 4) It is of immediate interest that the decrease in liver arginase after hypophysectomy occurs in spite of an enhanced protein catabolism[7] Furthermore, intense glycosuria and glyconeogenesis during alloxan diabetes are not accompanied by any change in the arginase activity of the rat liver[7]

[6] The addition of aqueous adrenal cortical extract to the homogenate of normal rat liver does not produce an increase in the "alkaline" phosphatase activity (preliminary experiments)

[7] Unpublished

The failure of thyroxine to affect liver arginase is in agreement with the negative results obtained by Lightbody and Kleinman (15) It is of interest that Fraenkel-Conrat *et al* (13) found that the thyrotropic hormone was ineffective in short term experiments but produced a small decrease in hypophysectomized rats after 10 days of treatment Similar results were obtained with thyroxine

The failure of any of the C_{11} steroids to influence the enzymes of the kidney of the normal rat is not surprising The small changes observed in the adrenalectomized rat (2, 4, 14) were in all probability restorative in nature

SUMMARY

Aqueous (beef) adrenal cortical extract, lipoextract (hog adrenals), and 11-dehydrocorticosterone acetate produced very marked increases in the "alkaline" (pH 9 8) phosphatase of the liver of fasted rats when injected eight times at hourly intervals The increase in enzyme activity did not parallel the degree of glyconeogenesis Thyroxine produced a marked depletion of liver glycogen and a decrease in the enzyme Epinephrine produced a tremendous deposition of liver glycogen but did not affect the activity of the enzyme

In none of the above treatments were the activities of the arginase and "acid" (pH 5 4) phosphatase of the liver or the enzymes of the kidney altered

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STUDIES ON THE METABOLISM OF RADIOACTIVE NICOTINIC ACID AND NICOTINAMIDE IN MICE*

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The use of isotopes as tracers, both stable and radioactive, has become a well established technique for the study of intermediate metabolism of many compounds of biological importance Recently the synthesis of radioactive nicotinic acid and nicotinamide (with C^{14} in the carboxyl group) has been reported by Murray, Foreman, and Langham (1)

In this paper the gross metabolism of radioactive nicotinic acid and nicotinamide in mice is discussed These results were obtained by determining the amount of radioactivity appearing in the exhaled air, urine, feces, and tissues as a function of time, following their administration by intraperitoneal injection

EXPERIMENTAL

The apparatus which was used is shown in Fig 1 Three mice were put into each of several cages, *G*, on glass screen, *K*, air (dried and freed of CO_2 by passage through drierite and ascarite) was drawn into the system through *I* at a rate of approximately 500 ml per minute as determined by a flow meter The exhaled CO_2 was collected in a train of three towers containing 20 per cent CO_2-free NaOH Urine was collected in *A* under toluene Feces were collected in *B* In order to minimize the contamination of urine and feces with food, *D* was inserted to trap most of the powdered food particles A single stick of mouse food $\frac{1}{2} \times 4$ inches was inserted into *E* on elevation *F*, which allowed animals to eat but prevented them from gnawing off any large particles and bringing them into the cage Water was supplied through *H* Funnel *C* gave good separation of feces and urine, each relatively uncontaminated with food

On Day 1 of the experiment 0 7 mg of radionicotinic acid (50,000 counts per second) was injected intraperitoneally into each of nine mice (Strain CF-1) which were then immediately put into metabolism cages (Fig 1)

* This document is based on work performed under contract No W-7405 eng 36 for the Atomic Energy Project, and the information covered therein will appear in Division V of the National nuclear energy series as part of the contribution of the Los Alamos Laboratory

Presented at the 113th meeting of the American Chemical Society

A second set of nine animals was injected with 0 7 mg each (50,000 counts per second) of radionicotinamide and similarly treated The β radiation from these injections was approximately 1 roentgen equivalent per day for the 1st day and much less on succeeding days because of the high excretion rate Urine, feces, and CO_2 were collected at various time intervals and C^{14} activity determined All measurements of radioactivity were made with the assembly described by Dauben *et al* (2)

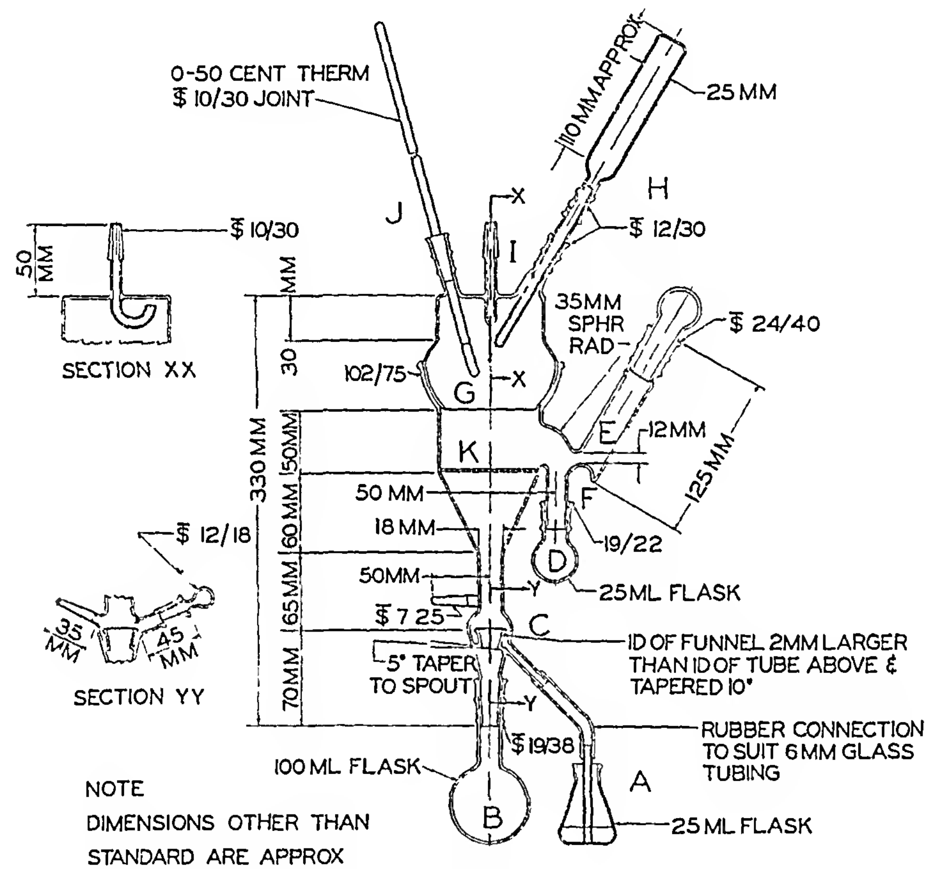

FIG 1 All glass metabolism cage for collecting urine, feces, and CO_2

The Geiger-Muller tube used had a thin mica window, 5 4 cm in diameter and 1 7 mg per sq cm thick The tube was filled to atmospheric pressure with helium saturated with alcohol at 3°

Urine samples were collected, diluted to 25 ml, and an aliquot plated directly on oxidized copper disks The measured activity was corrected for mass absorption with calibration curves developed by preparing plates of increasing amounts of biological fluids containing a constant amount of a water solution of radioactive nicotinic acid [1]

[1] Hogness, J R, Roth, L J, Leifer, E, and Langham, W, *J Am Chem Soc*, in press

Exhaled carbon dioxide was collected in 20 per cent NaOH and converted to $BaCO_3$. The $BaCO_3$ was precipitated onto oxidized copper disks, counted, and the results corrected for absorption by the method of Yankwich *et al.* (3).

The feces were dried in an oven at 96°, pulverized, and an aliquot extracted with water in a micro Soxhlet apparatus. More than 95 per cent of the C^{14} activity was removed in this manner. An aliquot of the water solution was plated directly and counted.

The data presented for urine, feces, and CO_2 are an average for nine mice.

A second series of mice was injected with similar doses of radionicotinic acid and radionicotinamide. The animals were sacrificed at various time

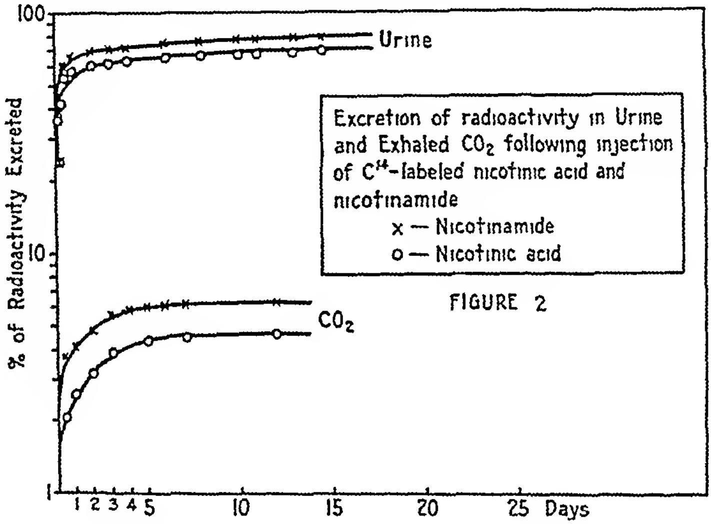

Fig. 2

intervals for purposes of tissue analysis. All tissues except blood were dried in an oven at 96°, they were then burned by standard procedures and the CO_2 formed in the combustion was absorbed in 10 per cent CO_2-free NaOH, precipitated as $BaCO_3$, and plated onto copper disks. Radioactivity was determined as above.

Blood samples were heparinized and centrifuged. The plasma was separated from the cells and the cells laked with distilled water. Radioactivity in plasma and laked cells was determined by plating the biological fluid directly on copper disks. Corrections for absorption were made according to specially developed absorption curves.

DISCUSSION

Fig. 2 shows the excretion of radioactivity in the urine and CO_2 for nicotinic acid and nicotinamide. Each point represents an average value for

the nine animals studied in each group On Day 1 there is a large excretion of radioactivity in both urine and CO_2 An initial injection of 0 7 mg of nicotinic acid, which exceeds the normal requirement, results in a large excretion of radioactivity in the first 24 hours The decrease in the slope of the urine curves which occurs after 24 hours is presumably due to the incorporation of the nicotinic acid and nicotinamide into the metabolic pool of the animal, whereas the initial rapid excretion may be unmetabolized injected material On the other hand, the rapid initial excretion of CO_2 represents normal metabolism or detoxification After the first 48 hours, when the nicotinic acid and nicotinamide are presumably in the form of coenzyme in the various tissues, a comparison of the ratio of radioactivity found in the CO_2 and urine would indicate that from 15 to 20 per cent of the

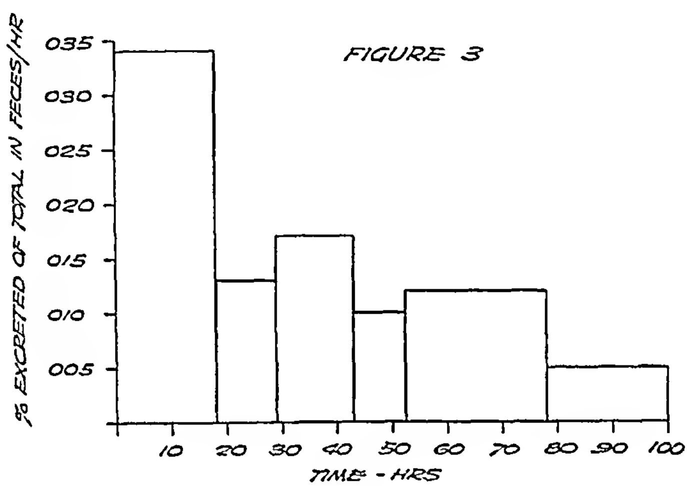

Fig 3

fixed nicotinic acid or nicotinamide is eliminated as CO_2 If one assumes that the radioactive CO_2 found in the first 48 hours is due only to the normal metabolic processes, it can be concluded that the total amount of fixed nicotinic acid or nicotinamide is approximately 40 per cent of the injected dose

It is to be noted that the urine curves are parallel, as are the CO_2 curves, indicating that nicotinic acid and nicotinamide enter into the same metabolic system This is confirmed by the results obtained on individual tissue analysis

Work on the identification of the various metabolic products in the urine is in progress

Fig 3 shows the radioactivity obtained by extracting the feces The data are plotted as a bar graph to emphasize the irregularities in excretion Because of the low activity in the feces and the likelihood of contamination

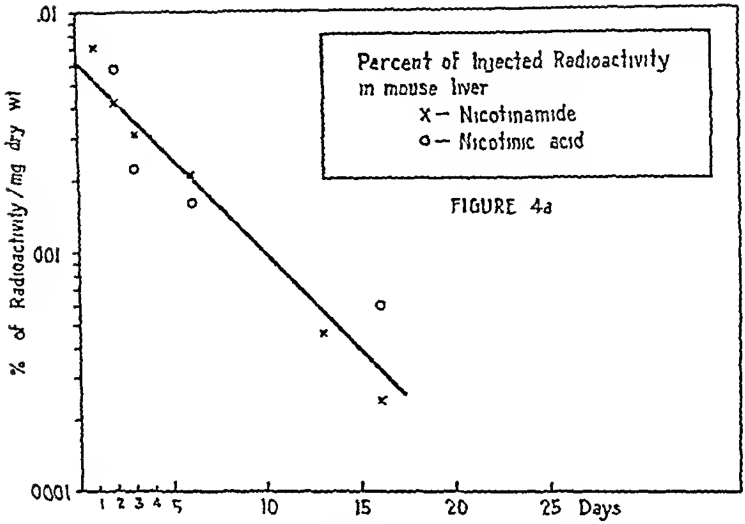

Fig 4, *a*

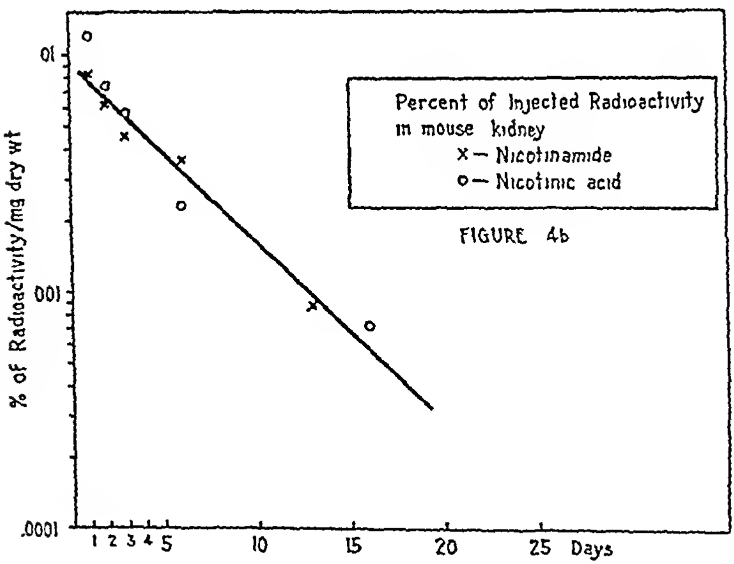

Fig 4, *b*

from the much more active urine, it is felt that little significance can be attached to these data If any fecal excretion of metabolites occurs, it is quite low

The activity in the various tissues resulting from uptake of the injected

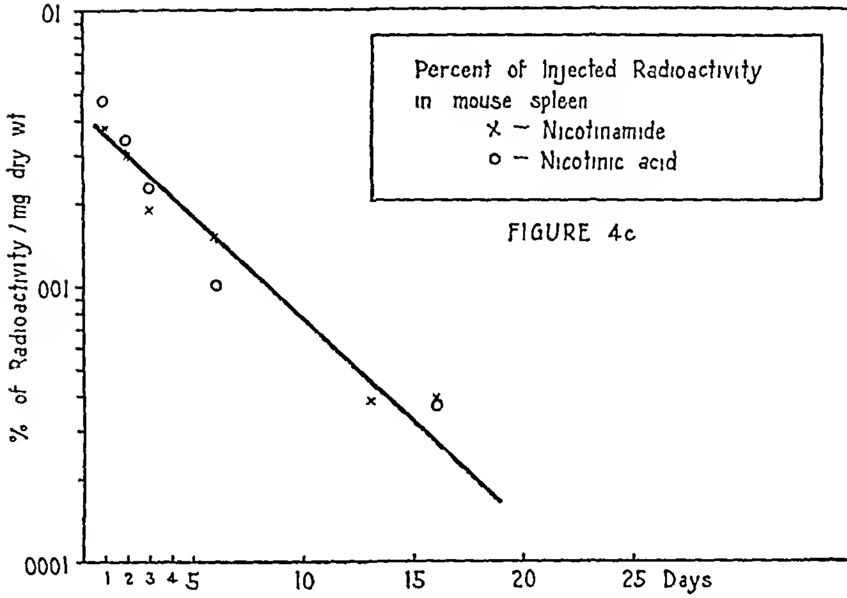

FIG 4, *c*

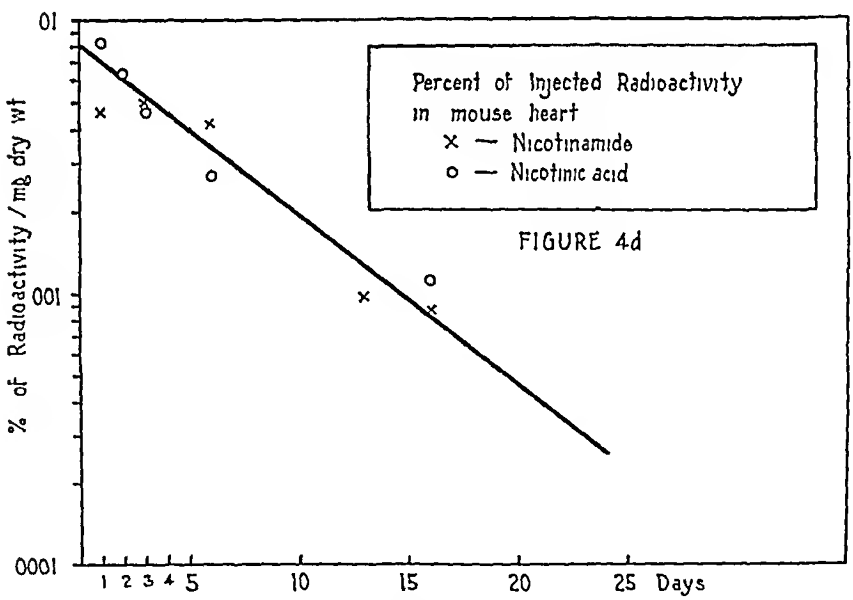

FIG 4, *d*

nicotinic acid and nicotinamide was determined at increasing time intervals by sacrificing the animals and preparing plates for counting as described above Fig 4, *a* through *h*, shows these data on a semilog plot

The results show that the uptake of radioactive nicotinic acid or nico-

tinamide varies with the different organs and is highest in kidney and lowest in erythrocytes No radioactivity was found in the plasma after the first

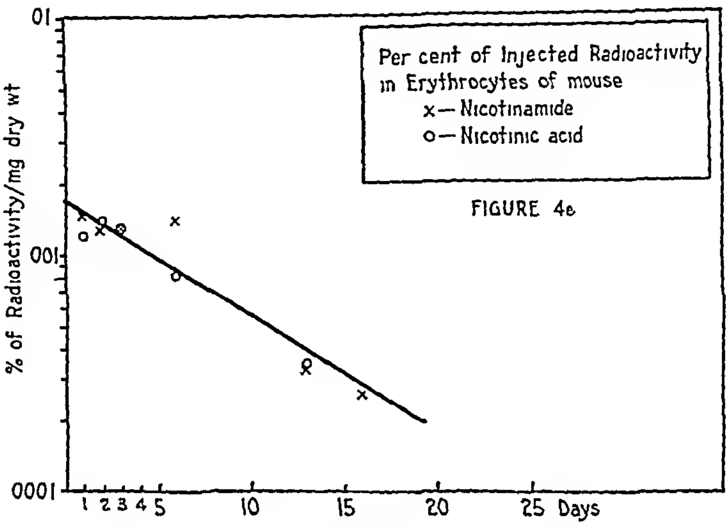

FIG 4, e

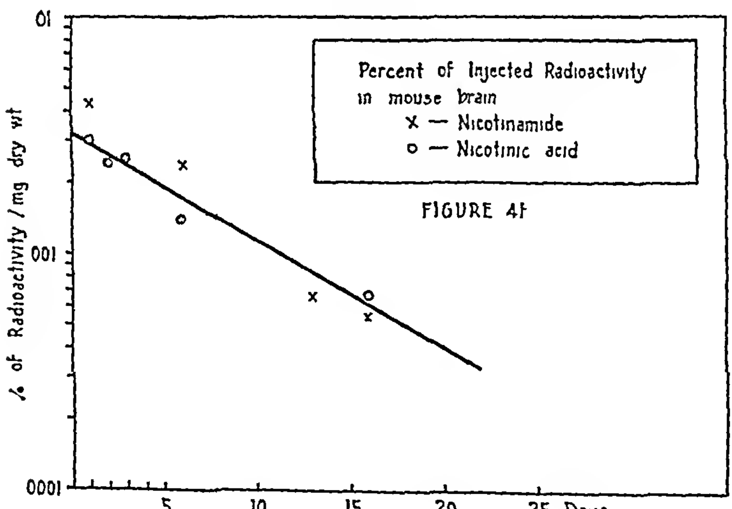

FIG 4, *f*

24 hours The excretion half times vary also with the different organs and are about 4 days in liver, kidney, and spleen, 5 days in cardiac muscle and

erythrocytes, and 8 days in brain, sternum, and skeletal muscle Presumably the major portion of the radioactivity determined was in the form of

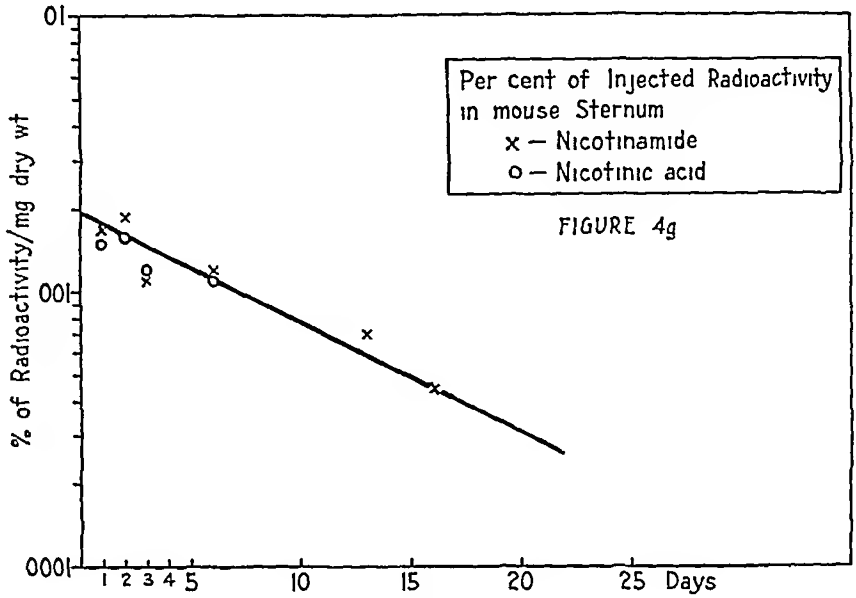

Fig 4, *g*

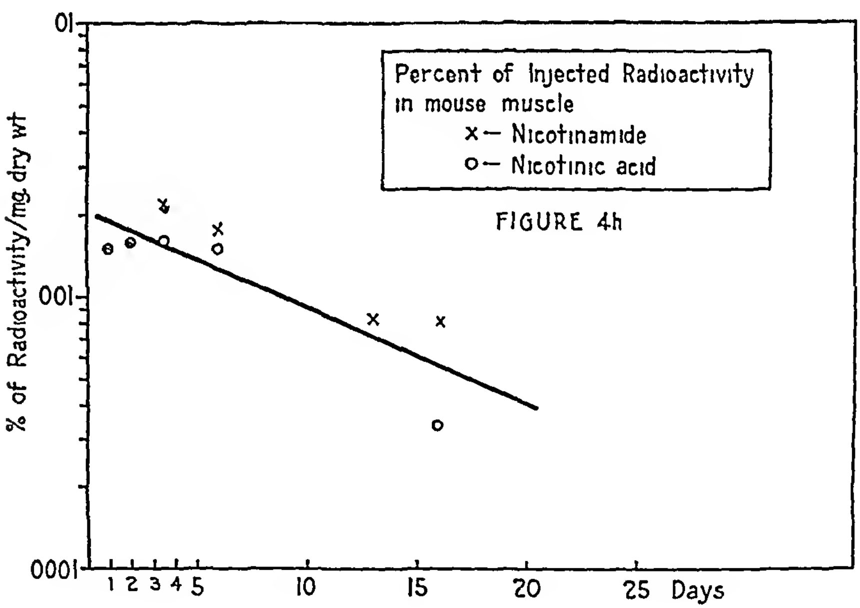

Fig 4, *h*

coenzyme, as it has been shown repeatedly that nicotinic acid and nicotinamide administered *in vivo* result in an increase in tissue coenzyme

SUMMARY

1 Gross metabolism studies on nicotinic acid and nicotinamide with use of compounds tagged with C^{14} in the carboxyl group are presented

2 It is shown that of the radioactivity fixed in the tissues about 15 per cent of the C^{14} in the carboxyl group appears as exhaled CO_2 Presumably this occurs as a result of decarboxylation, but there may also be ring rupture followed by decarboxylation In any case, the metabolites which have been isolated to date have all contained a carboxyl group in the 3 position of the pyridine ring The fact that nicotinic acid and nicotinamide are decarboxylated should stimulate the search for additional metabolic products

3 The gross metabolism of nicotinic acid and nicotinamide is identical in the mouse

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INTRACELLULAR DISTRIBUTION OF ENZYMES

III THE OXIDATION OF OCTANOIC ACID BY RAT LIVER FRACTIONS*

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Insoluble residues prepared by low speed centrifugation of tissue homogenates made in isotonic saline have recently been used to study the enzyme systems for the oxidation of fatty acids (6–9, 11), the oxidation of the acids of the Krebs tricarboxylic acid cycle (3), oxidative phosphorylation (10), *p*-aminohippuric acid synthesis (2), and citrulline synthesis (1) Our own studies on the differential centrifugation of rat tissue homogenates have shown that isotonic saline produces extensive agglutination of the mitochondria with the result that the fraction obtained at low speed was a mixture of all of the nuclei and 40 to 80 per cent of the mitochondria of the homogenate in addition to any cells that had not been broken during the homogenization (5, 14) It is, therefore, of interest to determine whether the enzymatic reactions mentioned above are associated with the nuclei, the mitochondria, or the unbroken cells, or whether a combination of two or more fractions is necessary for enzymatic activity Such a study was made possible by the finding that agglutination was greatly decreased when the homogenates were made in isotonic or hypertonic sucrose solutions (5) Thus a more efficient separation of nuclei and mitochondria was possible in the sucrose homogenates than had hitherto been possible in the saline homogenates

The present report describes the study of the oxidation of octanoic acid by fractions obtained from rat liver and shows that the oxidation of this fatty acid is associated mainly with the mitochondria [1]

Materials and Methods

Preparation of Livers—Stock rats originally of the Sprague-Dawley strain obtained from the Holtzman Company, Madison, were fasted overnight to eliminate glycogen from the liver The rats were killed by decapitation and the livers were removed and chilled in ice-cold isotonic KCl After

* This work was aided by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council

[1] This finding confirms the preliminary report of Kennedy and Lehninger (6) No comparison is possible between their results and those obtained in the present work because the tissue concentration used by these authors was not stated

cooling, the livers were blotted, weighed, and homogenized (12) in ice-cold isotonic sucrose (8.5 gm. of sucrose per 100 ml. of solution). Isotonic sucrose was used rather than the hypertonic sucrose necessary to maintain the morphological integrity of the mitochondria (5), because it was found in preliminary experiments that preparations made in the hypertonic solutions had a much lower rate of fatty acid oxidation than did preparations made in isotonic sucrose. The latter gave activities in the same range as those reported by Potter (11).

Differential Centrifugation of Rat Liver Homogenate[2]—10 ml. of homogenate (1 gm. of rat liver per 10 ml. of homogenate) were centrifuged for 10 minutes at $600g$ to sediment the nuclei. The supernatant was removed and the sediment was washed twice by resuspension in 2.5 ml. portions of isotonic sucrose and recentrifugation at $600g$ for 10 minutes. The washed sediment was made up to 2.5 ml. with isotonic sucrose and labeled the nuclear fraction, N_W. The supernatant and washings from the nuclear fraction were combined and centrifuged 10 minutes at $8500g$ to sediment the mitochondria. The mitochondria were washed twice by resuspension in 2.5 ml. of isotonic sucrose and recentrifugation at $8500g$ for 10 minutes. The washed mitochondria were made up to 2.5 ml. with isotonic sucrose and labeled the mitochondrial fraction, M_W. The supernatant and washings from the mitochondrial fraction were combined and centrifuged for 60 minutes at $18,000g$ to sediment the submicroscopic particles. The submicroscopic particles were washed once by resuspension in 2.5 ml. of isotonic sucrose and recentrifugation at $18,000g$ for 60 minutes. The washed particles were made up to 2.5 ml. with isotonic sucrose and labeled the submicroscopic particles, P_W. The supernatant and washing from the submicroscopic particles were combined, made up to 20 ml., and labeled the supernatant, S_2.

Measurement of "Octanoxidase"[3] *Activity*—Optimum concentrations of the components of the octanoxidase system were determined by Potter (11) and were those employed in this paper. The measurements of oxygen uptake were made in the Warburg apparatus at 38°. The flasks contained 0.01 M potassium phosphate buffer, pH 7.4, 0.01 M potassium malonate,[4]

[2] All of the centrifugations were made at 0° in the International refrigerated centrifuge PR-1. The centrifugations at $600g$ were made with the horizontal yoke No. 269 and those at $8500g$ and $18000g$ were made with the multispeed attachment and the No. 295 conical head. The centrifugal forces designated refer to the centers of the tubes employed.

[3] The term "octanoxidase" will be used to refer to that system of enzymes involved in the oxidation of octanoic acid.

[4] It has been claimed that malonate produces varying degrees of inhibition of octanoxidase activity depending upon the strain of rat employed (9). Since we have

pH 7 4, 0 0133 M magnesium chloride, 0 001 M adenosine triphosphate (K salt), 0 0000133 M cytochrome *c*, 0 028 M NaCl, 0 001 M potassium octanoate, and 0 8 ml of liver homogenate or an equivalent amount of each fraction The final volume was 3 0 ml Readings of oxygen uptake were made at intervals of 5 minutes after a 6 minute equilibration period The rate of oxygen uptake was constant for a period of at least 20 minutes and was proportional to the tissue concentration The oxygen uptakes over the 20 minute period were averaged

Analytical Methods—Desoxypentosenucleic acid (DNA) and pentosenucleic acid (PNA) were extracted with hot trichloroacetic acid and were estimated by means of colorimetric reactions (13) Total nitrogen was determined colorimetrically after acid digestion (16)

Reagents—The potassium octanoate was obtained by neutralizing octanoic acid (Eastman) that had been distilled *in vacuo* in an all-glass apparatus Adenosine triphosphate (Ba salt) was generously supplied by Dr G A LePage and was prepared as previously described (16)

Results

Distribution of Nucleic Acids and Total Nitrogen—The distribution of nucleic acids and total nitrogen in the rat liver fractions is given in Table I The nuclear fraction was found to contain 13 3 per cent of the nitrogen of the homogenate, 13 8 per cent of the PNA, and 99 per cent of the DNA The latter result indicated that the nuclei had apparently been quantitatively recovered in this fraction Microscopical examination of the nuclear fraction showed large numbers of intact nuclei both free and clumped together as well as a few unbroken cells and some free mitochondria, while examination of the other fractions failed to show either nuclei or nuclear fragments The mitochondria fraction contained 26 9 per cent of the nitrogen of the homogenate and 7 2 per cent of the PNA The submicroscopic particles on the other hand contained 52 6 per cent of the PNA of the homogenate and only 18 8 per cent of the nitrogen Thus the PNA concentration in the submicroscopic particles was much higher than in any of the other rat liver fractions (76 7 γ of PNA per mg of N) The supernatant contained the largest proportion of the nitrogen of the homogenate (41 3 per cent) and a considerable amount of PNA (23 2 per cent of the homogenate) The PNA concentration in the supernatant (15 1 γ per mg of N) was low, however, indicating that the removal of the submicroscopic particles in the previous step of the centrifugation had

not experienced significant inhibition in the presence of malonate (Table III), it may be questioned whether the inhibitions observed (9) may not be related to the use of *water* suspensions of the enzyme or to the presence of malate in their assay system

effectively removed that portion of the liver homogenate containing the highest concentration of PNA

Distribution of Octanoxidase Activity—The results of the measurements of octanoxidase activity are presented in Table II With the exception of the

TABLE I

Nucleic Acid and Nitrogen Content of Fractions Isolated from Homogenates of Rat Liver in Isotonic Sucrose

The average values are given in bold-faced type

The figures in parentheses represent the range of the values in the four experiments reported

Fraction	Nitrogen		DNA phosphorus*			PNA phosphorus*		
	Total†	Fraction of homogenate	Total†	Fraction of homogenate	Per mg nitrogen	Total†	Fraction of homogenate	Per mg nitrogen
	γ	*per cent*	γ	*per cent*	γ	γ	*per cent*	γ
Homogenate	**3018** (2967–3048)	(100)	**27 1** (24 9–28 6)	(100)	9 0	**82 8**	(100)	27 4
Nw	**402** (366–430)	13 3	**26 8** (24 8–28 5)	99 0	66 6	**11 4**	13 8	28 3
Mw	**811** (700–890)	26 9				**6 0**	7 2	7 4
Pw	**568** (500–715)	18 8				**43 5**	52 6	76 7
S_2	**1247** (1174–1330)	41 3				**19 2**	23 2	15 4
Recovery		100 3		99 0			96 8	
No of determinations	4		4			2		

* Phosphorus calculated from pentosenucleic acid and desoxypentosenucleic acid determinations

† Per 100 mg of fresh liver or its equivalent

supernatant the fractions were tested separately as well as in all possible combinations The supernatant was tested only separately The results show that when tested separately only the mitochondrial fraction possessed octanoxidase activity The activity of the mitochondrial fraction (67 c mm of oxygen absorbed per 10 minutes) was increased to 79 c mm per 10 minutes when either the nuclear fraction or the submicroscopic particles

were added and to 85 c mm per 10 minutes when both of these fractions were added simultaneously to the mitochondria The reason for the increased activity observed when these fractions were added to the mitochondrial fraction is not known An explanation for these results may be possible when all the enzymes involved in the octanoxidase system are known and can be tested separately in the fractionation procedure

TABLE II

Oxidation of Potassium Octanoate by Fractions Isolated from Homogenates of Rat Liver in Isotonic Sucrose

The average values are given in bold-faced type and represent the experiments for which the nitrogen and nucleic acid content were reported in Table I

Fraction	Oxygen uptake*			
	+ Octanoate	− Octanoate	Corrected for endogenous	Fraction of homogenate
	c mm per 10 min	*c.mm per 10 min*	*c.mm per 10 min*	*per cent*
Homogenate	**107** (100–117)	**24** (8–31)	**83** (78–92)	(100)
Nw	**2 3** (0–9 1)		**2 3**	**2 8**
Mw	**67** (64–70)		**67** (64–70)	**81** (71–87)
Pw	**0** (0)		**0**	**0**
Nw + Mw	**79** (72–86)		**79**	**95**
Nw + Pw	**1 1** (0–4 4)		**1 1**	**1 3**
Mw + Pw	**79** (54–94)		**79**	**95**
Nw + Mw + Pw	**86** (69–94)	**1 1** (0–3 0)	**84** (69–94)	**101** (90–105)
S_1	**5 6** (3 4–8 3)	**8 1** (5 6–11 7)		

* Per 100 mg of fresh liver or its equivalent

As pointed out in the first paper of this series, one of the fundamental principles that must be observed in fractionation studies, if the results of the assays are to have any significance, is that each fraction must be assayed for enzymic activity and that the total activity of the fractions must closely approximate the activity of the original homogenate Some difficulty was encountered in adhering to this principle in the study of the octanoxidase system It is apparent from Table II that the activity of the homogenate (107 c mm per 10 minutes) is considerably greater than the activity of the

recombined nuclear, mitochondrial, and submicroscopic particle fractions (85 c mm) It was observed, however, that the homogenate had a high endogenous uptake of oxygen (24 c mm), while these three fractions either separately or in combination had negligible endogenous oxygen uptake The supernatant on the other hand showed a considerable endogenous oxygen uptake (8 1 c mm) When the endogenous uptake was subtracted from the uptake in the presence of octanoate, the activity of the homogenate (83 c mm per 10 minutes) agreed closely with the activity of the combined nuclear, mitochondrial, and submicroscopic particle fractions (84 c mm per 10 minutes) Inasmuch as the activity of the three recombined fractions agreed reasonably well with the corrected activity of the homogenate in each of four experiments, it was concluded that this correction was valid

TABLE III

Effect of Repeated Sedimentation of Mitochondria from Isotonic Sucrose on Their Ability to Oxidize Octanoate and on Their Nitrogen Content

Each figure represents the average of duplicate measurements

Mitochondria preparation	Oxygen uptake*	Nitrogen*
	c mm per 10 min	γ
M (sedimented once)	56 6 (58 3)	780
M_1 (" twice)	52 5 (57 5)	723
M_2 (" 3 times)	54 8 (59 5)	723
M_3 (" 4 times)	55 6 (54 5)	726

* Per the equivalent of 100 mg of fresh liver The figures in parentheses represent single measurements of the octanoxidase activities when malonate was omitted from the assay system

even though all of the endogenous uptake was not recovered in the fractions On the basis of the corrected values for the homogenate the mitochondria possessed an octanoxidase activity that was 81 per cent as great as that of the homogenate Thus it can be concluded that the major portion of the activity of this enzyme system was associated with the mitochondria

Effect of Repeated Sedimentation on Octanoxidase Activity of Rat Liver Mitochondria—Since the results described in the preceding paragraph indicated that the major portion of the octanoxidase activity of rat liver homogenates was associated with the mitochondria, it was of interest to study the effect of repeated sedimentation of the mitochondria from isotonic sucrose on their octanoxidase activity The results are presented in Table III The data show that the total octanoxidase activity remained essentially constant after as many as four sedimentations The nitrogen content of the mitochondria fraction decreased from 780 γ in the first sedimentation

to 723 γ after the second sedimentation Further sedimentation did not alter the nitrogen content appreciably The loss of nitrogen that occurred during the second sedimentation was probably due to the removal of soluble material or of particles smaller than the mitochondria

DISCUSSION

The oxidation of fatty acids by rat liver homogenates is believed to involve two major types of reactions (1) oxidation of the fatty acid to acetoacetic acid by successive oxidative removal of 2-carbon fragments and their subsequent recondensation, or (2) oxidation of the fatty acid to CO_2 and H_2O by condensation of the 2-carbon units with oxalacetic acid to form acids of the Krebs tricarboxylic acid cycle (8) Lehninger (8) has presented data to show that octanoic acid is quantitatively oxidized in the presence of adenosine triphosphate and magnesium ions to acetoacetic acid by the insoluble material obtained by low speed centrifugation of rat liver homogenates and Potter (11) has presented confirmatory evidence in support of this data Since the measurements of octanoxidase activity in this paper were made under conditions strictly comparable to those of these authors, it may be concluded that the oxidation of octanoic acid to acetoacetic acid is associated almost exclusively with the mitochondria of the rat liver homogenate (Table II) With respect to the localization of the enzymes involved in the further oxidation of fatty acid (the enzymes of the Krebs cycle), it may be stated that several of the individual enzymes have already been demonstrated to be almost exclusively associated with the large granules or mitochondria of rat liver Thus the enzymes succinic dehydrogenase, cytochrome oxidase, and cytochrome *c* have been found to be associated with these particulate components of the liver cell (4, 5, 14, 15) The assay of the oxalacetic acid-oxidase system, which appears to be an over-all measure of the enzymes of the Krebs cycle, has also been made on rat liver fractions A large fraction of the oxalacetic acid oxidase activity of the homogenate was recovered in the mitochondrial fraction The results of these studies will be described in a subsequent paper

The demonstration that the octanoxidase activity of rat liver homogenates was associated exclusively with the mitochondria and not with the nuclei would have been impossible if a cytochemical search had not been instituted for a medium in which cells could be disrupted without concomitant agglutination of the mitochondria Fortunately such a medium was discovered in the non-electrolyte sucrose (5) In the absence of such a study, the biochemist might have been tempted to conclude that oxidation of fatty acids by the insoluble material obtained from tissue homogenates was associated with the nuclei, inasmuch as the size and the physical and chemical properties of the latter overshadow those of the mitochondria

One instance can already be cited in which an enzymatic function has been assigned to the nuclei present in such a mixed preparation (1) The present report shows that the oxidation of octanoic acid does not require nuclei and demonstrates how the more exact localization of the other enzymatic activities associated with insoluble tissue residues (1–3, 10) may be determined

SUMMARY

1 Rat liver homogenates in isotonic sucrose were separated by differential centrifugation into nuclear, mitochondrial, submicroscopic particle and supernatant fractions

2 The distribution of pentosenucleic acid (PNA) and desoxypentosenucleic acid (DNA) was determined in the fractions The entire DNA of the homogenate was recovered in the nuclear fraction PNA was found in all of the fractions, but in the submicroscopic particle fraction the PNA concentration per mg of nitrogen was 2 8 times as great as in the homogenate and 52 6 per cent of the PNA of the homogenate was recovered in this fraction

3 The distribution of the activity of the enzyme system oxidizing octanoic acid was also studied It was found that the major portion of the activity of this enzyme system was recovered in the mitochondria fraction

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ON HEPARIN MONOSULFURIC ACID*

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The chemical nature of pure heparin has attracted much attention Even before its chemistry was known attempts were made to isolate crystalline derivatives of it (1, 2) When heparin was found to be a polysulfuric acid ester of a polysaccharide, showing a resemblance to chondroitinsulfuric acid (3), serious attempts were made to crystallize its barium salt (4) This salt has, in fact, a crystalline appearance

Many findings, however, raise considerable doubt regarding its homogeneity When the barium salt is recrystallized from a 20 per cent acetic acid solution, an inactivation occurs, probably due to depolymerization, without loss of the crystalline appearance (5) The barium salts contain other cations besides barium (6) The sulfur content and the biological activity vary in different preparations, particularly if heparin samples from different animals are compared (7, 8) It seemed to one of us (9) most plausible to consider the heparin as a polysaccharide extensively esterified with sulfuric acid, and the ordinary heparin preparations as inhomogeneous mixtures of mono-, di-, and trisulfuric acids Brucine salts of heparin fractions with a sulfur content corresponding to that of all these three acids were also isolated (10) All of them showed an anticoagulant effect, which increased within the same animal species with increasing sulfur content The possibility of obtaining a homogeneous polysaccharide polysulfuric ester seemed therefore to be very remote In fact, Kuizenga and Spaulding (11), in 1943, separated different fractions with different sulfur contents from a barium salt assumed to be crystalline As will be reported further on, we have made the same observation

So far, attention was focused only on the fractions having the highest sulfur content and the strongest anticoagulant activity The sulfur content of some fractions also reached the figures calculated for a trisulfuric acid, 13 6 per cent for the sodium salt (12) and 10 8 per cent for the barium salt (7, 9, 13) A sodium salt of such a fraction from ox liver with 130 Toronto units per mg was, moreover, in 1942 selected by the Department of Biological Standards of the National Institute for Medical Research, London, to serve as the provisional international heparin standard

* This investigation was aided by grants from the Knut and Alice Wallenberg Foundation, Stockholm

The question then arises whether the more soluble fractions of the brucine and barium salts with a lower sulfur content and a weaker anticoagulant activity are chemical entities *per se* or only mixtures of one or several indifferent polysaccharides and the trisulfuric acid ester We have now analyzed these fractions more closely and found that after removal of at least all the trisulfuric acid they still have an anticoagulant effect and a sulfur content which corresponds in the main fraction to that of the monosulfuric acid of heparin

Fractionation of Easily Soluble Barium Salts

In the Charles and Scott procedure of 1933 (2) for the large scale extraction of heparin from ox liver and ox lungs, the alkaline extract is acidified with sulfuric acid Heparin and chondroitinsulfuric acid are thereby precipitated together with the proteins After complete deproteinization, the heparin is precipitated as a barium salt insoluble in water The soluble barium salts of the mother liquor are precipitated with alcohol

Although there is a marked difference in the solubility of the two main fractions of barium salts, a certain amount of heparin tri- and disulfuric esters could nevertheless be assumed to be present in the mother liquor among the easily soluble barium salts, thus explaining their anticoagulant activity Steps were therefore taken to remove any admixture of ordinary heparin that might be present

To a 20 per cent aqueous solution of the easily soluble barium salts barium chloride was added to 5 per cent, followed by 0 1 volume of a hot saturated solution of barium hydroxide After standing for 24 hours in the ice box, the precipitate was removed It usually comprised 2 per cent of the weight of the easily soluble barium salts Another fraction of similar size was removed by adding 0 05 volume of alcohol Both these small fractions contained about 6 to 7 per cent sulfur and about 15 heparin units per mg of dry substance The preparations from liver and lungs behaved similarly in these respects The admixture of trisulfuric acid must consequently have been insignificant, and after removal of these fractions this acid is still less likely to occur as an impurity in the barium salts to be discussed below

The absence of less soluble barium salts was easily demonstrated by the following procedure

If half the volume of a saturated solution of barium chloride was added to a neutral 5 per cent solution of the easily soluble barium salts which had been precipitated with 0 1 volume of alcohol (Samples A and B in Table I), no opalescence was seen, not even after standing for 24 hours in the ice box If the solution contained 0 05 per cent of the sodium salt of heparin with 11 5 per cent sulfur and 110 units per mg simultaneously, an opalescence

was observed on adding the barium chloride On the following day there was a slight precipitate adhering to the bottom of the test-tube Twice the amount of heparin, or 0 1 per cent, immediately gave a strong opalescence and on the following day an ordinary precipitate If heparin had been added to 0 25 per cent, a precipitate formed immediately when the barium chloride solution was added

The same occurred if protamine, free of sulfate ions, was added to these solutions 0 5 mg of protamine base added to 0 5 ml of a 5 per cent solution of the easily soluble barium salts gave an opalescence If the solution contained 0 1 or 0 25 per cent of heparin simultaneously, a heavy precipitate formed on adding the protamine

In these tests a 5 per cent solution of the easily soluble barium salts gave no precipitate with barium chloride, whereas such a low concentration of heparin as 0 05 or 0 1 per cent could easily be demonstrated The anticoagulant effect of the easily soluble barium salts, 10 to 16 units per mg, would have demanded an admixture of barium salt of ordinary heparin, making up about 10 per cent of their weight It is therefore evident that these polysaccharide fractions with only one sulfuric acid group for each disaccharide unit also have an anticoagulant effect of their own

The less soluble barium salts of ordinary heparin thus having been removed, the easily soluble barium salts were precipitated with alcohol from the alkaline solution containing 5 per cent of barium chloride, a first fraction being obtained with 0 1 volume of alcohol and the remainder with 1 volume This separation into two fractions proved to be very valuable The barium salt of the lung polysaccharide which precipitated with 0 1 volume of alcohol behaved in quite a different way from the fraction obtained with 1 volume The former exerted an anticoagulant activity, contained glucosamine, and was dextrorotatory, whereas the latter was biologically inactive and only weakly dextrorotatory It consisted of chondroitinsulfuric acid

The amount of soluble barium salts obtained after removal of ordinary heparin is different when one is working with samples from liver and from lung In the liver it is somewhat larger than the amount of sodium salt of heparin recovered from the insoluble barium salt, the proportions being 1 2 1 With lung the yield is smaller, the soluble barium salts comprising only a third of the weight of the heparin Furthermore, the easily soluble barium salts from these two sources also differ as to their composition, chondroitinsulfuric acid making up about a third of the samples from lung

The barium salts thus obtained were reprecipitated twice with alcohol, the second time after neutralization of the solution with hydrochloric acid Finally they were dissolved in a small volume of water and kept in the cold overnight Two fractions were thereby separated, Sample A, insoluble, and Sample B, soluble (see Table I), both being analyzed separately

Before analysis, all the barium salts were once more dissolved in a small volume of water and dialyzed against distilled water in cellophane sacs The results of the fractionation are given in Table I For comparison the theoretical figures for the nitrogen, sulfur, and ash content of the barium salt of the heparin monosulfuric acid are given in Table I

Analysis of Easily Soluble Barium Salts

Sulfur—The sulfur content of the main fraction of the easily soluble barium salts both from lung and liver, which was precipitated with 0 1

TABLE I

Fractionation of Easily Soluble Barium Salts

	Air dry substance	Dry substance			Heparin in mg dry substance	$[\alpha]^{20}_{D}$,* dry substance
		Ash	N	S		
	gm	*per cent*	*per cent*	*per cent*	*units*	*degrees*
Barium salt of heparin monosulfuric acid, $C_{14}H_{19}SO_{14}NBa$, mol wt 594 58		39 2	2 36	5 38		
Ox lung Crude easily soluble Ba salts	1000	33 0		5 25	10–11	
0 1 volume alcohol, Sample A	210	37 8	2 37†	6 67	16	+45 8
0 1 " " " B	127	39 6	2 61	5 42	10	+49 4
1 volume alcohol	230	33 1	2 41	4 44	1 2	+0 62
Ox liver Crude easily soluble Ba salts	1000	35 2		5 15	18–20	
0 1 volume alcohol, Sample A	14	41 9		6 30	16	
0 1 " " " B	505	39 6	5 43	5 69	18 (?)	
1 volume alcohol	92	35 3	3 90	4 13	7	

* 2 per cent in water

† 5 per cent of the nitrogen occurred as NH_2 nitrogen

volume of alcohol, was about that calculated for a heparin monosulfuric acid (see Table I) Sample B from lung contained 5 42 per cent of sulfur and from liver 5 69 per cent (calculated, 5 38 per cent) Fraction A, which precipitated at a low temperature, had a somewhat higher sulfur content, 6 67 and 6 30 per cent, as was also to be expected for the less soluble fraction Evidently, it contained an admixture of disulfuric acid of heparin polysaccharide

Amino Sugar—On acid hydrolysis 37 per cent of the calculated amount of amino sugar was isolated as pure crystalline glucosamine hydrochloride The high yield of glucosamine thus isolated almost excludes any considerable admixture of other amino sugars Consequently, the amino sugar is glucosamine, as in ordinary heparin (14)

Isolation of Glucosamine—30 gm of air-dry substance of Sample B (Table I, from lung) were hydrolyzed 7 hours in 500 ml of 20 per cent hydrochloric acid by heating under a reflux on a metal bath Hydrochloric acid was removed by repeated evaporations *in vacuo* to almost dryness, the last time after decolorizing with norit The dry residue was treated with 3 ml of hot normal hydrochloric acid to which 50 ml of methyl alcohol were added The insoluble residue, weighing 3 1 gm, proved to be pure crystalline glucosamine hydrochloride A 2 per cent solution gave after 2 minutes $[\alpha]_D^{20} = +100°$ and on the following day $+71°$ From the methyl alcohol another lot of crystals weighing 0 120 gm was obtained when acetone was added Total yield, 3 22 gm of glucosamine hydrochloride or 13 4 per cent of the dry substance of Sample B, Table I Consequently, the yield of glucosamine hydrochloride was 37 per cent of the theoretical (36 2 per cent)

In another hydrolysis, 1 87 gm of glucosamine hydrochloride was obtained from 20 gm of air-dry substance of Sample B, or almost the same yield, this time following hydrolysis in the presence of 10 gm of stannous chloride, as recommended by Levene

Exactly the same yield of crystalline glucosamine hydrochloride, 37 per cent of the theoretical, was obtained from 30 gm of air-dry substance, as in Table I Consequently, the amino sugar of the heparin monosulfuric acid is glucosamine as is that of the trisulfuric acid

The well known difficulty of obtaining galactosamine from chondroitinsulfuric acid was also experienced here

Simultaneously with the above hydrolyses of Samples A and B, 30 gm of air-dry substance of the last fraction of barium salts from lung, which had been precipitated with 1 volume of alcohol, were hydrolyzed without stannous chloride, and 20 gm after addition of stannous chloride, when both were submitted to exactly the same procedure as described above In none of these hydrolyses were any crystals obtained on adding methyl alcohol On adding acetone in both instances an insoluble sticky mass precipitated, a chondrosinc polymer, very familiar to everyone who has attempted to isolate chondrosamine from chondroitinsulfuric acid This detail, together with the low optical activity and the lack of stronger anticoagulant activity, made it quite evident that this *fraction of the easily soluble barium salts from lung consisted almost exclusively of chondroitinsulfuric acid* It also behaved like this acid on acid hydrolysis, and against the Schiff reagent after oxidation with periodic acid

A quantitative analysis for *uronic acid*, with the technique applied by Jorpes and Bergström in 1937 (15), gave 25 0, 30 0, and 30 0 (mean 28 35) per cent (calculated, 32 6 per cent)

An *acetyl determination* according to the method of Lumieux and Purves (16) with chromic acid oxidation gave 7 8, 7 31, and 7 8 (mean 7 5) per cent acetic acid (calculated 10 1 per cent)

Conclusions—The organic skeleton of the polysaccharide with one sulfuric acid group for each disaccharide unit thus consists of 1 molecule of amino sugar, glucosamine, 1 molecule of uronic acid, and 1 molecule of acetic acid

Because of the anticoagulant activity, 10 to 16 heparin units per mg of dry substance, and the similarity to heparin, the substance is assumed to be a monosulfuric ester of the heparin polysaccharide

The fraction of the easily soluble barium salts which precipitated with 1 volume of alcohol consisted, in the preparation from lung, almost exclusively of chondroitinsulfuric acid It made up a third of the weight of the purified, easily soluble barium salts It was practically devoid of anticoagulant activity, had a very low dextrorotation, and gave no glucosamine after acid hydrolysis The corresponding fraction of the easily soluble barium salts from cattle liver, however, showed a similarity to the monosulfuric ester, although it had a somewhat lower sulfur content It made up only 9 per cent of the weight of the crude, easily soluble barium salts The difference between these two preparations, from lung and liver respectively, was very clearly demonstrated through their rate of hydrolysis in 7 5 per cent (by volume) sulfuric acid and through their behavior in the fuchsin-sulfurous acid test after periodate oxidation The preparation from lung behaved in these respects like chondroitinsulfuric acid, and that from liver like monosulfuric ester of heparin

Acid Hydrolysis for Differentiating Polysaccharides

The anticoagulant activity of heparin is lost when a solution of the barium salt is heated repeatedly to 65° in 20 per cent acetic acid (5), probably owing to a depolymerization The sulfuric acid is also very easily split off, about 60 per cent of it being hydrolyzed in 5 minutes and all of it in 1 hour on boiling with 2 N hydrochloric acid The carbohydrate skeleton, however, is fairly resistant to acid hydrolysis, practically no reducing substance being liberated during 60 minutes boiling in 7 5 per cent (by volume) sulfuric acid This remarkable property of the heparin polysaccharide was observed when the behavior of heparin on acid hydrolysis was compared with that of other similar polysaccharides (15) The chondroitinsulfuric acid and the easily soluble fractions of the brucine salts of heparin hydrolyzed much more easily

We have found this principle of characterizing a polysaccharide very useful All the samples of the heparin monosulfuric acid from liver and lung (Samples 1 to 5, Fig 1) showed the same characteristic rate of hydrolysis

Heparin monosulfuric acid hydrolyzes more easily than di- and trisulfuric acids but with considerably lower speed than chondroitinsulfuric acid This indicates a difference in the internal structures of chondroitinsulfuric

acid and heparin monosulfuric acid The same circumstance is furthermore demonstrated by their different response to the Schiff reagent, fuchsin-sulfurous acid, after periodate oxidation, the latter giving a strong reaction, while chondroitinsulfuric acid gave practically none (17)

Heparin monosulfuric acid can also be differentiated from hyaluronic acid, which is hydrolyzed with even greater speed than chondroitinsulfuric acid *Heparin monosulfuric acid is consequently not a monosulfuric ester of hyaluronic acid*

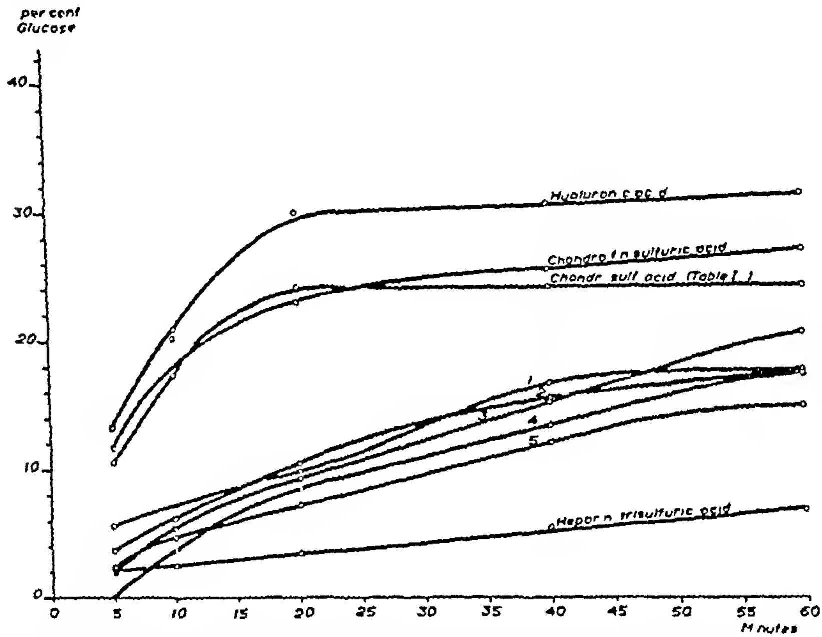

FIG 1 Reducing substance, calculated as glucose in per cent of the organic material liberated during hydrolysis in 7 5 per cent (by volume) sulfuric acid The figures on the curves refer to sample numbers

The most soluble fraction of the easily soluble barium salts of lung heparin behaved in this respect like chondroitinsulfuric acid, whereas that from the liver hydrolyzed like the heparin monosulfuric acid

Is Heparin Monosulfuric Acid Preformed in Tissues?—The first question which arises is whether the polysaccharide with low sulfur content and weak anticoagulant activity extracted from ox liver and ox lung together with ordinary heparin really occurs as such in the living organisms, or whether it is a degradation product of heparin The question is by no means easy to answer Evidently it could result from the action of sulfatases from the heparin already in the living cells The occurrence of a heparinase has also been discussed by Jaques (8)

This question cannot be answered without further analytical work along the same lines as those applied by Jorpes, Holmgren, and Wilander in 1937 (see Jorpes (9) pp 57-67), when they demonstrated that there is a very close relationship between the number of mast-cells in the different organs of different animals and their content of heparin

The next question is whether the monosulfuric acid is formed from heparin through the extractions process This question has been discussed earlier ((12) p 209) Tryptic digestion does not break down any heparin Heparin submitted to the same treatment as the lungs and the liver with an ammoniacal ammonium sulfate solution of pH 9 to 9 4 could be recovered unchanged, although a quantitative yield was not to be expected The sulfate groups, however, are rather easily hydrolyzed From heparin trisulfuric acid 33 per cent of the sulfur is split off at 100° in 0 1 N sodium hydroxide during 5 minutes, 36 per cent in 15 minutes, 54 per cent in 1 hour, and 66 per cent in 4 hours After 24 hours boiling in 5 N sodium hydroxide only 72 per cent is split off In an alkaline solution one sulfate group is thus very easily removed from the trisulfuric acid, the second more slowly, and the third with the utmost difficulty The sulfuric acid is, however, in alkaline solution, removed from the monosulfuric acid with about the same speed The monosulfuric ester can therefore not arise from di- and tri sulfuric esters in the course of alkaline extraction

Also, in a weakly acid solution, the sulfate groups are liberated at about the same speed from the mono- as from the di- and trisulfuric esters

Against this background the question of the chemical nature of heparin can more easily be discussed Fractions with the composition of a tri sulfuric acid have earlier been isolated and described Here we describe a monosulfuric ester of a polysaccharide, in many respects resembling heparin Ordinary heparin which precipitates as an insoluble barium salt from water is neither a trisulfuric acid nor a monosulfuric acid but a mixture of tri- and di- and possibly also some monosulfuric ester Even a tetrasulfuric ester might conceivably occur, as can be concluded from the following experiment We obtained from 74 gm of barium salt, "recrystallized" twice from 10 per cent acetic acid after heating to 60° through repeated dissolving in hot water, a number of fractions which after transformation into sodium salts gave the following yield of sulfur 15 83 gm 13 6 per cent, 2 14 gm 10 88 per cent, 3 36 gm 8 23 per cent, 6 24 gm 7 60 per cent, 7 36 gm 4 48 per cent

The high yield of the sodium salt with 13 6 per cent sulfur, the theoretical figure for the sodium trisulfuric acid of heparin, makes an admixture of a tetrasulfuric acid in this sample very probable So far, however, we have no conclusive evidence as to the occurrence of this acid

DISCUSSION

In discussing the possibilities at hand against the background of the analytical findings, one feels almost inclined not to speak of the different mono-, di-, and trisulfuric esters but to consider the heparin as a polysaccharide more or less esterified with sulfuric acid, in which all the different disaccharide units of one and the same molecular complex need not necessarily be esterified to the same degree The accumulation of sulfate groups determines the solubility of the barium salts and improves the anticoagulant activity There are samples with about three sulfate groups for each disaccharide unit, samples with only one group and intermediates, probably a mixture of di- and trisulfuric acid esters The occurrence of a tetrasulfuric acid of the same polysaccharide is very likely, as is also the occurrence of the sulfur-free heparin polysaccharide itself in the animal body

SUMMARY

The main fraction of the easily soluble barium salts remaining in the mother liquor when protein-free heparin from ox liver and ox lung is precipitated as an insoluble barium salt has the composition of a heparin monosulfuric acid It contains equal parts of glucosamine, a uronic acid, acetic acid, and sulfuric acid It is dextrorotatory, the barium salt having an optical rotation of approximately +50°

The preparations are practically free of heparin di- and trisulfuric acids and have an anticoagulant activity, in the form of the barium salt, between 10 and 16 provisional international heparin units per mg

The rate of hydrolysis at 100° in 7 5 per cent (by volume) sulfuric acid distinctly differentiates this polysaccharide from the chondroitinsulfuric acid which hydrolyzes much faster, and from the hyaluronic acid which hydrolyzes even more rapidly

The yield of this fraction from ox liver almost equals that of heparin, but that from lung makes up only a fifth

All of the heparin preparations are more or less inhomogeneous This applies to the strongest ones, with approximately three sulfate groups for each disaccharide unit, as well as to the monosulfuric acid It is questionable whether any chemically well defined heparin samples can be obtained

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THE FUCHSIN-SULFUROUS ACID TEST AFTER PERIODATE OXIDATION OF HEPARIN AND ALLIED POLYSACCHARIDES*

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Oxidation with periodic acid affords a possibility of drawing conclusions regarding the internal structure of some polysaccharides Since two adjacent hydroxyl groups are required, some compounds such as heparin trisulfuric acid, in which sulfate groups occupy three of the four hydroxyl groups assumed to be present, will not react with periodic acid Ordinary heparin has therefore been found not to consume periodate (1) It also gives a negative reaction with fuchsin-sulfurous acid after treatment with periodic acid A fraction of the heparin polysaccharide with a lower sulfur content, the heparin monosulfuric acid described in the preceding paper (2), on the contrary, consumes iodate and gives a strongly positive reaction in the color test

In applying the color test to different polysaccharides related to heparin it was found that chondroitinsulfuric acid, which has the same elementary composition as heparin monosulfuric acid, gives a negative test This result was somewhat unexpected The different behavior of the two related polysaccharides can, however, easily be explained

Depending on the linkages between the two main components of these mucopolysaccharides, the amino sugar and the uronic acid, the sulfate-free polysaccharide will have two adjacent hydroxyl groups either in both of the components of the disaccharide unit (*cf* (2) Fig 1, upper structure), in one (lower structure), or in none of them When a hydroxyl group becomes esterified with sulfuric acid, one α-glycol structure is eliminated

The different behavior of the two related compounds, heparin monosulfuric acid and chondroitinsulfuric acid, to periodic acid consequently discloses a difference in the internal structure of the two polysaccharides Furthermore, the same was demonstrated when the polysaccharides were submitted to acid hydrolysis in 7 5 per cent (by volume) sulfuric acid (*cf* (2) lower structure, Fig 1)

The reaction with periodic acid can also be used for staining polysaccharides in animal and plant tissues, as suggested recently by Hotchkiss

* This investigation was aided by grants from the Knut and Alice Wallenberg Foundation, Stockholm

(3), the reaction products, the aldehydes, giving a red color with fuchsin-sulfurous acid Since ordinary heparin does not react and heparin monosulfuric acid gives a positive reaction, we applied this color reaction to the tissue mast-cells, the heparinocytes, which are known to produce heparin, hoping to find a positive reaction indicating the presence of the monosulfuric acid in these cells Actually, the granules of the heparinocytes of the subcutaneous tissue of the young rat readily gave a very intense red color with fuchsin-sulfurous acid after treatment of the tissue slices with periodic acid, but no color without this treatment The histological technique, however, is not yet fully elaborated and does not allow any final conclusions to be drawn Many sources of error must first be eliminated Thus, for ex-

$$\begin{matrix} HCOH \\ HCOH \end{matrix} + HJO_4 \rightarrow \begin{matrix} HCO \\ HCO \end{matrix} + HJO_3 + H_2O$$

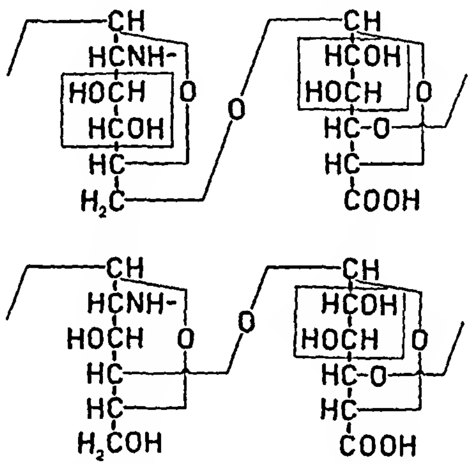

FIG 1 A glucosaminoglucuronoside with two groups (above) and one group (below) reacting with periodic acid

ample, oxidants such as chromic acid cannot be used for fixation of the material, and the fuchsin itself has a great affinity for the tissues The question of the occurrence of the heparin monosulfuric acid in the tissues will be studied further by us both chemically and histologically The resistance of the chondroitinsulfuric acid to periodate could easily be demonstrated histologically In staining sections from trachea with the Hotchkiss technique, the mucous cells acquired a bright red color from hyaluronic acid, whereas the cartilage remained completely unstained

EXPERIMENTAL

Consumption of Periodic Acid by Some Mucopolysaccharides

The consumption of periodic acid was measured according to the method of Fleury and Fatôme (4)

50 mg samples were dissolved in 5 ml of water to which 3 ml of a 0 11 N periodic acid solution were added The samples were left standing for 20 hours at room temperature After that time the excess of periodate was destroyed through addition of 10 ml of a 0 049 N arsenous acid solution Some potassium iodide was added and the arsenous acid not consumed was oxidized with 0 1 N iodine solution

The consumption of periodic acid by some polysaccharides related to heparin, calculated on 100 mg of ash-free organic substance, was as follows

	S per cent of dry substance	Periodic acid consumed, mg HIO_4
Hyaluronic acid, Na salt		54 6
Heparin monosulfuric acid, Ba salt	5 42	52 8
Chondroitinsulfuric " Ca "	3 91	16 3
Heparin trisulfuric " Na "	12 17	8 2

The consumption of periodic acid by heparin monosulfuric acid equals that of the hyaluronic acid as calculated on organic substance It is evident that heparin trisulfuric acid does not react with the periodic acid, the low figure found being partly due to the presence of pigments adhering to the heparin Chondroitinsulfuric acid consumes a certain smaller amount of periodic acid but not more than can be explained through the splitting off of sulfate groups The sulfur content of this preparation of chondroitinsulfuric acid was 3 91 per cent, whereas the calculated figure was 4 94 per cent About 20 per cent of the sulfates had been split off during preparation, thus liberating α-glycol groups

Color Test on Different Polysaccharides

The color reaction with fuchsin-sulfurous acid after periodate oxidation can, as well as the periodate consumption, be used as a qualitative test for glycol structures in sugars and for a rough quantitative estimation of polysaccharides One of us (B Å) elaborated a technique for the quantitative colorimetric test in which the excess of periodic acid was destroyed through ultraviolet irradiation before the fuchsin-sulfurous acid was added

The procedure was as follows (1) Periodic acid, 400 mg of H_5IO_6, and 135 mg of NaAc $3H_2O$ were dissolved in 50 ml of water, pH about 2, (2) 1 N HCl, (3) fuchsin-sulfurous solution made up as follows 2 gm of basic fuchsin dissolved in 400 ml of boiling water To the cooled and filtered solution 10 ml of 2 N hydrochloric acid were added and 4 gm of potassium metabisulfite, this was decolorized with 1 gm of charcoal Up to 10 ml or more of 2 N hydrochloric acid were added in small portions until, after the last addition, the mixture dried spontaneously in a thin film on a glass slide, without giving a pink color

Method—The substance was dissolved in 5 ml of water, and 3 ml of the

periodic acid solution were added, whereupon the sample was heated to boiling The solution was immediately irradiated by a 500 watt quartz lamp in a 125 ml quartz flask The lamp, 20 5 cm high, was placed 7 cm above the flask and surrounded by an aluminum cylinder having a diameter of 18 cm In order to prevent reduction of the periodic acid to iodine the lamp was not allowed to radiate with its full capacity On irradiation with

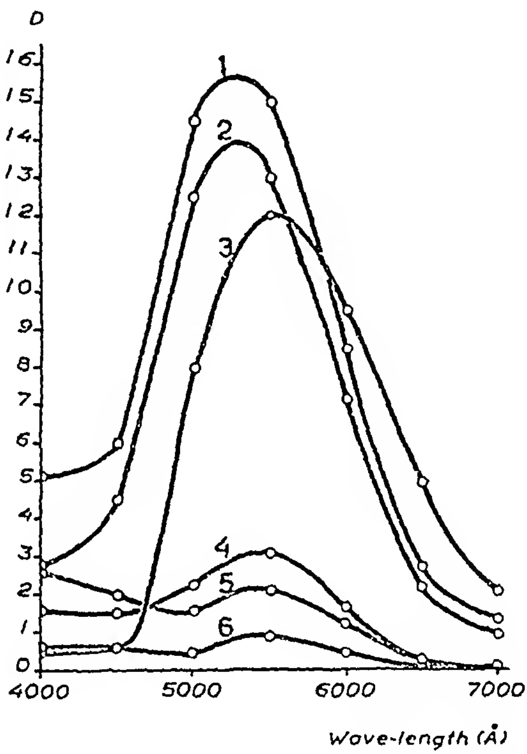

FIG 2 Color intensity in the fuchsin-sulfurous acid reaction after periodate oxidation of some polysaccharides related to heparin Air-dried samples Curve 1, hyaluronic acid, sodium salt, 50 mg , Curve 2, heparin monosulfuric acid, barium salt, 50 mg , Curve 3, galacturonic acid, 50 mg , Curve 4, chondroitinsulfuric acid, calcium salt, 100 mg , Curve 5, heparin trisulfuric acid, sodium salt, 50 mg , Curve 6, water

1 8 amperes during 8 minutes, no free iodine was produced and the heat was very slight After irradiation 2 ml of 1 N HCl and 8 drops of the Schiff reagent were added The color extinction was measured after 5 minutes in a Coleman spectrophotometer

The color intensity given in this reaction by the different mucopolysaccharides is shown in Fig 2 As is seen, heparin trisulfuric acid and chondroitinsulfuric acid do not react with periodate, whereas heparin monosulfuric acid and hyaluronic acid are easily oxidized

The color intensity obtained with hyaluronic acid is proportional to the

amount of substance present (Fig 3), a proportionality not to be found on analysis of heparin monosulfuric acid

DISCUSSION

Oxidation with periodic acid has revealed definite differences between heparin monosulfuric acid and chondroitinsulfuric acid There must be a

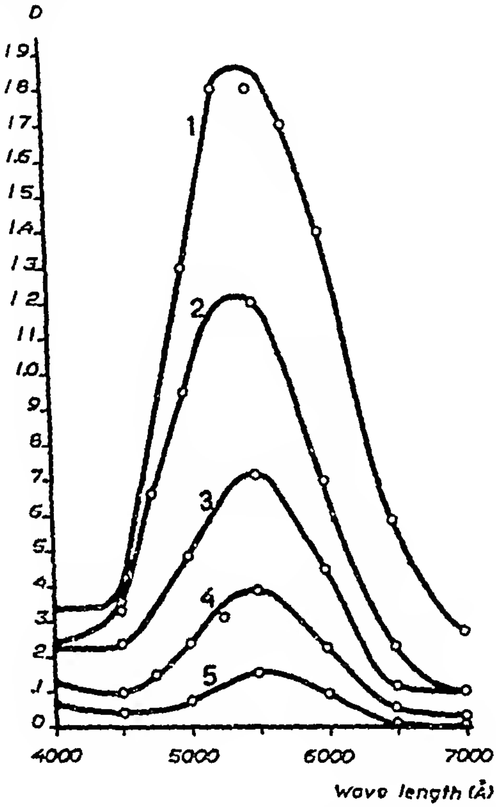

FIG 3 The color intensity of different amounts of hyaluronic acid (sodium salt) in the fuchsin sulfurous acid test after periodate oxidation Calculated on ash-free organic substance Curve 1, 30 mg , Curve 2, 20 mg , Curve 3, 10 mg , Curve 4, 5 mg , Curve 5, water

difference either in the linkages between the single sugar components or in the location of the sulfate group In accordance with this they also show a different behavior toward hydrolysis with acids In the chondroitin-sulfuric acid there cannot be more than one glycol unit with two adjacent hydroxyl groups The sulfuric acid is probably bound to one of these hydroxyl groups, because as long as chondroitinsulfuric acid is intact, as it is

in the cartilage, it does not react with periodic acid and gives a negative Schiff color reaction, whereas the ordinary preparations of chondroitinsulfuric acid consume a small amount of periodate This is probably due to a loss of sulfate groups during preparation, as is also evidenced through the low sulfur content

The Schiff reaction, if applied histologically, seems to enable us to demonstrate the presence of the heparin monosulfuric acid in tissues Thus the granules of the tissue mast-cells in the subcutaneous tissue of the young rat give a very strong fuchsin-sulfurous acid reaction, which could be due to the presence of this acid or some other heparin precursor

SUMMARY

Whereas ordinary heparin does not react with periodic acid, the heparin monosulfuric acid gives a color reaction with fuchsin-sulfurous acid after periodate oxidation

The granules of the mast-cells in the subcutaneous tissue of the rat are strongly colored with Schiff's reagent, which could indicate the presence of heparin monosulfuric acid or some other heparin precursor Chondroitin sulfuric acid is resistant to this reagent, the cartilage being completely unstained when treated with periodic acid and subsequently with fuchsin sulfurous acid This indicates a difference between chondroitinsulfuric acid and the heparin monosulfuric acid, either in the internal linkages between the two carbohydrate moieties or in the location of the sulfate groups

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THE METABOLISM OF PLUTONIUM IN RATS FOLLOWING INTRAMUSCULAR INJECTION*

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PLATES 1 AND 2

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The metabolism of plutonium in man has become a matter of concern with the development of the chain-reacting pile For this reason a portion of the first plutonium produced by the Clinton pile was set aside for animal studies in order to evacuate the hazards which plutonium might present to man In March, 1944, 11 mg of plutonium were made available to this laboratory for animal studies from the first production of the Clinton pile Later studies were undertaken at the Metallurgical Laboratory, University of Chicago (1), and at Los Alamos (2)

Plutonium is produced by the neutron irradiation of U^{238}, the U^{239} initially formed decays by the emission of a negative β-particle The Ne^{239} created by this process in turn decays by negative β-particle emission to form Pu^{239} This isotope of plutonium, having a half life of 24,000 years, decays by the release of α-particles to V^{235}, which occurs in nature and has a half life of 7×10^8 years

Methods

Preparation of Material—The plutonium was prepared in the three valence states for injection by Professor R Connick and his colleagues, working under the direction of Dean W M Latimer in the College of Chemistry

The +3 valence state was prepared by the reduction of $PuCl_4$ by SO_2 in 0 47 N HCl The +4 plutonium was prepared prior to the intramuscular injection by first precipitating the +4 hydroxide from the original $PuCl_4$ solution and dissolving the precipitate in 0 47 N HNO_3 The + 6 plutonium was prepared by oxidation of the $PuO_2(NO_3)_2$ with dichromate The plutonium was precipitated as sodium plutonyl acetate, the preparation dissolved in HCl, and then precipitated as $NaPuO_2Ac_3$ The precipitate was again dissolved in HCl and the plutonium reprecipitated with NaOH Finally, this precipitate was dissolved in 0 45 N HCl

*This work was performed under contract No W-7405-eng-48-A with the Manhattan District, United States Army

The solutions containing the three separate valence states of plutonium were tested qualitatively by a spectrophotometric method and each was found to contain less than 20 per cent of plutonium in the form of valence states other than the one specifically desired These stock solutions contained approximately 2 mg of plutonium per cc Immediately before injection the solutions were diluted with sufficient isotonic saline to bring the pH to 2 5, and they contained approximately 15 γ of plutonium per cc

Administration to Rats—Owing to nutritional and metabolic similarities to man, the animal chosen for this work was the rat The three plutonium solutions, as described above, were immediately administered to rats In order to avoid undue plating of the plutonium on the syringes and needles used for injection, they were washed with 0 01 N HCl before use In spite of this precaution, considerable difficulty was experienced with plating, especially with respect to Pu in the +4 state However, the equipment *used for injection was assayed for plutonium in order that the actual dose* of Pu administered to the rats could be determined

Twelve groups of three animals each were injected with approximately 15 γ of Pu intramuscularly in the left leg, the Pu solutions being employed in the three valence states described above Following this, groups of rats were sacrificed at 4, 16, 64, and 256 days after administration of plutonium in the +3, +4, and +6 valence states

Three groups of rats were given plutonium in its three valence states orally and sacrificed 4 days later

The excreta were collected from all groups at daily intervals In order to reduce the rather laborious plutonium assays as much as possible, the urine and feces were pooled for the following intervals, 0 to 2 days, 2 to 4 days, 4 to 8 days, 8 to 16 days, and every 8 days thereafter When the animals were sacrificed, the organs removed included the liver, kidney, testes, spleen, muscle, skin, stomach, large and small intestine together with the cecum, bone, lung, brain, and blood The left hind leg, which was the site of intramuscular administration, was removed and assayed separately in order to determine the percentage of plutonium remaining unabsorbed Testes, spleen, muscle removed from the uninjected right leg, stomach, small and large intestines, lungs, brain, and 3 to 5 cc of blood obtained by heart puncture were pooled for each group In the case of bone, the uninjected right leg bones were assayed separately from the rest of the skeleton The skinned carcass, which consisted of the rest of the skeleton, muscle, fat, and blood, was dried and ashed as a unit The skeleton was separated from this by sifting the ash through a fine screen The plutonium content of both of these portions was determined The plutonium present in this ash, not included in the skeleton, is listed under "balance" in Tables I, III, and V after the calculated plutonium content of the muscle system of the animal had been subtracted from it

The tissues were assayed in the following manner Immediately after the animals were sacrificed, the organs and tissues were removed and weighed while wet During the first part of the experimental program with plutonium, all of the tissues were ashed in an electric muffle following a preliminary drying at 150° Several determinations were done in which a known amount of plutonium in its three valence states was added to inactive animal ash, which was brought up to a temperature of 500° and maintained at this level for 24 hours No measurable amount of plutonium was lost from this ashing method by volatilization

In later studies the tissues were wet ashed with concentrated HNO_3 and 30 per cent H_2O_2 When the ashing was completed, all of the acid was boiled off, leaving a white ash Following either wet or dry ashing, the ash was dissolved in a solution which was 2 N HNO_3 and 0 2 M hydroxylamine The final concentration of animal ash in the nitric acid hydroxylamine solution was 20 mg per cc 0 5 cc of the solution was used for the assay of plutonium Duplicate assays were made for every tissue and the details of the analytical procedure were as follows

500 γ of $La(NO_3)_3$ in 25 microliters were added to 0 5 cc of a solution of tissue ash in a 2 cc centrifuge cone (Because of the presence of α-emitting contaminants in some of the $La(NO_3)_3$ available, the latter must be checked for α emitters by running blanks on each batch received The radioactive contamination present is believed to be actinium, whose chemical properties resemble closely those of lanthanum, as well as plutonium in the +4 state)

0 2 cc of 6 N HF was added and the mixture stirred with a platinum rod The cone was then centrifuged at 6000 R P M in a small centrifuge for 30 seconds The cone was removed and the walls washed down with the supernatant solution The mixture was centrifuged for 3 minutes and the supernatant solution discarded The precipitate of LaF_3 containing the plutonium was then dissolved in concentrated nitric acid and transferred to a platinum dish 1 inch in diameter The cone was washed with a small amount of water, and this was also added to the dish 2 drops of 6 N HF were added to the solution in the platinum dish in order to reprecipitate the LaF_3 and the plutonium

The dish containing the precipitate was gently warmed on a hot-plate until dry and then flamed to a dull red heat for a few seconds

The precipitate was found to form a very thin even film which was quite adherent to the platinum The α-particles from these samples were counted, each sample being counted twice It was found that the LaF_3 precipitated from the diluted ash samples of bone, feces, and urine was somewhat more bulky than would be expected from the amount of lanthanum used as a carrier A large series of α-particle measurements was made from the ash of these three types of material, and a relatively constant

degree of self-absorption of the α-particles was found which ranged from 18 to 22 per cent for the feces and bone and 10 per cent for the urine. Owing to the fact that the remainder of this extraneous material in the LaF_3 precipitate would have necessitated a slow and laborious purification procedure, the appropriate correction factor was applied to these samples.

TABLE I

Deposition of +3 Plutonium in Tissues of Rat after Intramuscular Administration into Left Leg

The values given are in per cent of the dose. Average values for three rats at each time interval.

	4 days		16 days		64 days		256 days	
	per cent per organ	*per cent per gm*	*per cent per organ*	*per cent per gm*	*per cent per organ*	*per cent per gm*	*per cent per organ*	*per cent per gm*
Lungs	0.05	0.04	0.06	0.05	0.09	0.05	0.07	0.05
Spleen	0.08	0.13	0.09	0.15	0.22	0.40	0.21	0.42
Blood	0.45	0.04	0.20	0.02	0.10	0.01		
Liver	1.12	0.14	0.87	0.14	1.71	0.30	0.93	0.12
Kidney	0.30	0.18	0.10	0.07	0.27	0.19	0.31	0.17
Brain	<0.01*	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Stomach	0.06	0.02	0.03	0.02	<0.01	<0.01	0.09	0.03
Intestines	0.25	<0.01	0.23	0.03	0.08	<0.01	0.12	<0.01
Bone	10.8	0.64	18.7	1.13	30.3	2.09	30.6	1.97
Muscle	0.50	<0.01	0.76	<0.01	2.74	0.03	1.66	0.02
Balance	2.16		1.81		1.78		1.37	
Left leg	77.1		68.0		39.7		23.7	
Skin	0.61	0.03	0.62	0.03	0.49	0.02	0.20	<0.01
Gonads			0.06	0.02				
Tail					1.68	0.24		
Urine	0.11		0.19		0.97		1.12	
Feces	1.53		0.74		19.5		30.8	
Actual recovery	95.1		92.5		99.6		91.2	

* Although samples in this and the succeeding tables marked <0.01 per cent had measurable amounts of activity, the activities were of a low order and are probably not significant to more than two decimal places.

Bone radioautographs were prepared from undecalcified femur sections by techniques described elsewhere (3). These were made from animals sacrificed at intervals ranging from 8 to 256 days after intramuscular administration. Doses ranging from 15 to 25 γ were used.

Results

The average value of absorption of plutonium in each of its three valence states from the gastrointestinal tract was found to be 0.007 per cent. This

value may be even lower, since it is predicated primarily upon the limits of the sensitivity of the counting apparatus used and the number of micrograms of plutonium available for these studies

Data are presented in Tables I and II which give the relative deposition of plutonium in the tissues of the rat when administered in the +3 state These studies were carried out for 256 days after administration of the plutonium, as were those involving the +4 and +6 valence states It can be seen from an examination of Table I that +3 plutonium is only partly absorbed from the injection site, 77 per cent remaining at 4 days and 23 per

TABLE II

Deposition of +3 Plutonium in Tissues of Rat Corrected for Recovery and for Unabsorbed Balance at Injection Site

Average values for three rats at each time interval

	4 days		16 days		64 days		256 days	
	per cent per organ	*per cent per gm*	*per cent per organ*	*per cent per gm*	*per cent per organ*	*per cent per gm*	*per cent per organ*	*per cent per gm*
Lungs	0 32	0 25	0 26	0 22	0 15	0 09	0 11	0 08
Spleen	0 50	0 82	0 40	0 66	0 38	0 69	0 32	0 64
Blood	2 84	0 25	0 88	0 09	0 17	0 02		
Liver	7 06	0 88	3 84	0 62	2 94	0 52	1 41	0 18
Kidney	1 89	1 13	0 44	0 31	0 46	0 33	0 47	0 26
Brain	0 03	0 02	0 03	0 02	0 02	<0 01	<0 01	<0 01
Stomach	0 38	0 23	0 13	0 09	0 01	0 01	0 14	0 05
Intestines	1 58	0 06	1 02	0 13	0 14	<0 01	0 18	<0 01
Bone	68 1	4 04	82 6	4 99	52 1	3 59	46 3	2 98
Muscle	3 15	0 04	3 36	0 04	4 71	0 05	2 51	0 03
Skin	3 85	0 19	2 74	0 13	0 84	0 03	0 30	0 01
Gonads			0 26	0 09				
Tail					2 89	0 41		
Urine	0 69		0 84		1 67		1 69	
Feces	9 65		3 27		33 5		46 6	

cent at 256 days The largest portion of the material absorbed is deposited in the skeleton Liver, kidney, and spleen were the only other tissues examined which contained relatively large concentrations of plutonium The excretion of plutonium occurred primarily in the feces In Table II the same data are presented with corrections[1] for recovery and absorption from the injection site

[1] These corrected values were obtained by calculating the relative amounts of plutonium present in all of the tissues and excreta, exclusive of the plutonium remaining unabsorbed in the left hind leg, and the amount of plutonium in the balance less the calculated quantity present in the blood, muscle, and carcass This type of extrapolation, which attempts to correct for the amount of unabsorbed plutonium

Tables III and V demonstrate the deposition of plutonium in the tissues of the rat when administered as the +4 and +6 valence states The corrected values are given in Tables IV and VI These data are similar to those obtained for plutonium in the +3 valence state, demonstrating major deposition in the skeleton Of all of the soft tissues, the liver, kidney, and spleen show the highest affinity for plutonium per gm However, their concentration of plutonium per gm averaged from one-fifth to one-tenth that of bone The corresponding concentration of plutonium in the other soft tissues was very much less

TABLE III

Deposition of +4 Plutonium in Tissues of Rat after Intramuscular Administration into Left Leg

The values given are in per cent of the dose Average values for three rats at each time interval

	4 days		16 days		64 days		256 days	
	per cent per organ	*per cent per gm*	*per cent per organ*	*per cent per gm*	*per cent per organ*	*per cent per gm*	*per cent per organ*	*per cent per gm*
Lungs	<0 01	<0 01	0 01	0 01	0 03	0 02	0 02	0 01
Spleen	<0 01	<0 01	0 01	0 03	0 03	0 07	0 05	0 12
Blood	0 05	<0 01	0 01	0 01	0 04	0 01		
Liver	0 08	<0 01	0 19	0 03	0 56	0 08	0 41	0 04
Kidney	0 03	0 02	0 08	0 04	0 10	0 05	0 07	0 03
Brain	<0 01	<0 01	<0 01	<0 01	<0 01	<0 01	<0 01	<0 01
Stomach	<0 01	<0 01	<0 01	<0 01	<0 01	<0 01	<0 01	<0 01
Intestines	0 05	<0 01	0 05	<0 01	0 11	<0 01	0 07	<0 01
Bone	1 44	0 08	2 18	0 14	12 9	0 84	11 5	0 60
Muscle	0 03	<0 01	0 08	<0 01	0 63	<0 01	0 59	<0 01
Balance	2 35		1 05		1 68		0 28	
Left leg	95 8		87 6		68 1		66 8	
Skin	<0 01	<0 01	0 06	<0 01	0 17	<0 01	0 21	<0 01
Gonads			<0 01	<0 01	0 05	0 02	0 06	0 02
Tail					0 84	0 13		
Urine	0 01		0 04		0 39		0 43	
Feces	0 11		0 43		4 70		9 42	
Actual recovery	100 0		91 8		90 3		89 9	

present, appears to be the best method of indicating the true metabolic behavior of this substance The observed quantity of plutonium in the balance, less that estimated to be present in the blood and muscle of the carcass, is thought to represent the amount of the injected solution which extravasated beyond the point of amputation and remained unabsorbed

For all groups it will be noted that the corrected data show that there is no apparent and significant difference in either the distribution or excretion of plutonium in its three valence states This is demonstrated in Text-fig 1 which shows the relative proportions of the absorbed plutonium in the three valence states in the excreta and in the skeleton Their similarity suggests that plutonium exists in the body in the same valence state, regardless of the valence state in which it was administered

TABLE IV

Deposition of +4 Plutonium in Tissues of Rat Corrected for Recovery and for Unabsorbed Balance at Injection Site

Average values for three rats at each time interval

	4 days		16 days		64 days		256 days	
	per cent per organ	*per cent per gm*	*per cent per organ*	*per cent per gm*	*per cent per organ*	*per cent per gm*	*per cent per organ*	*per cent per gm*
Lungs	0 22	0 17	0 19	0 16	0 15	0 10	0 09	0 04
Spleen	0 33	0 50	0 32	0 95	0 15	0 34	0 22	0 53
Blood	2 76	0 22	0 19	0 01	0 19	0 01		
Liver	4 40	0 39	6 03	0 95	2 72	0 39	1 79	0 18
Kidney	1 66	1 10	2 54	1 27	0 49	0 24	0 31	0 13
Brain	0 04	0 03	0 02	0 02	<0 01	<0 01	0 04	0 03
Stomach	0 11	0 05	0 13	0 06	0 05	0 03	0 04	0 02
Intestines	2 76	0 28	1 59	0 16	0 15	0 04	0 31	0 02
Bone	79 3	4 40	69 3	4 44	62 7	4 09	50 3	2 63
Muscle	1 66	0 02	2 54	0 03	3 06	0 03	2 58	0 02
Skin	0 22	0 03	1 91	0 06	0 83	0 03	0 92	0 02
Gonads			0 32	0 10	0 24	0 10	0 26	0 09
Tail					4 09	0 63		
Urine	0 56		1 27		1 90		1 88	
Feces	6 06		13 6		22 9		41 2	

However, a very great difference in the amounts of plutonium absorbed from the site of injection was noted for the three valence states The greatest degree of absorption was observed for +6 plutonium, the least for +4 plutonium, and +3 was intermediate in character These dissimilar rates of uptake from the injection site are shown in Text-fig 2

Radioautographs

Radioautographs of adult rat femurs were made at periods ranging from 8 to 256 days after plutonium administration All of these showed the deposition of plutonium on bone surfaces, *i e*, in the region of the periosteal and endosteal bone surfaces, and on the endosteal covering of the trabecular

Table V

Deposition of +6 Plutonium in Tissues of Rat after Intramuscular Administration into Left Leg

The values given are in per cent of the dose, average values for three rats at each time interval

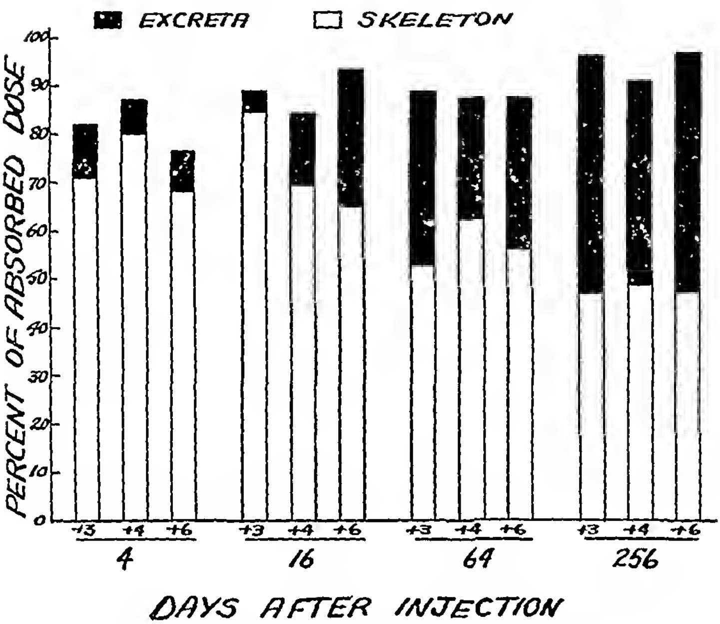
	4 days		16 days		64 days		256 days	
	per cent per organ	*per cent per gm*	*per cent per organ*	*per cent per gm*	*per cent per organ*	*per cent per gm*	*per cent per organ*	*per cent per gm*
Lungs	0.08	0.06	0.10	0.08	0.09	0.06	0.05	0.03
Spleen	0.10	0.16	0.22	0.40	0.28	0.47	0.20	0.34
Blood	0.86	0.07	0.21	0.02	0.11	<0.01		
Liver	3.58	0.47	1.89	0.34	2.24	0.30	1.09	0.13
Kidney	0.50	0.28	0.47	0.28	0.91	0.48	0.20	0.12
Brain	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Stomach	0.17	0.05	0.06	0.03	0.08	0.04	0.03	0.01
Intestines	0.59	0.03	0.50	0.05	0.26	0.02	0.09	<0.01
Bone	17.2	0.83	43.7	1.78	28.7	1.96	34.6	2.31
Muscle	0.65	0.01	1.56	0.02	0.83	0.01	0.73	0.01
Balance	4.73		1.50		3.57		2.71	
Left leg	70.4		30.0		33.6		12.6	
Skin	0.56	0.02	0.60	0.02	0.60	0.02	0.26	0.01
Gonads			0.10	0.04				
Tail					1.72	0.28		
Urine	0.10		5.51		2.30		3.28	
Feces	1.95		13.4		14.1		32.4	
Actual recovery	101.5		99.8		90.4		88.3	

Table VI

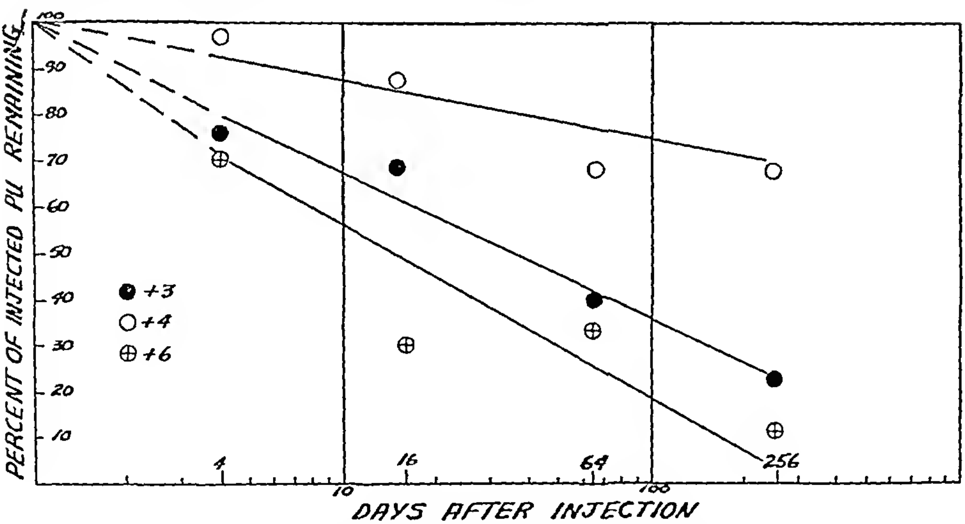
Deposition of +6 Plutonium in Tissues of Rat Corrected for Recovery and for Unabsorbed Balance at Injection Site

Average values for three rats at each time interval

	4 days		16 days		64 days		256 days	
	per cent per organ	*per cent per gm*	*per cent per organ*	*per cent per gm*	*per cent per organ*	*per cent per gm*	*per cent per organ*	*per cent per gm*
Lungs	0.30	0.23	0.15	0.12	0.17	0.11	0.07	0.04
Spleen	0.38	0.61	0.32	0.59	0.53	0.88	0.27	0.47
Blood	3.27	0.27	0.31	0.03	0.21	0.02		
Liver	13.6	1.78	2.77	0.50	4.21	0.56	1.49	0.18
Kidney	1.90	1.06	0.69	0.41	1.71	0.90	0.27	0.16
Brain	0.03	0.02	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Stomach	0.65	0.19	0.09	0.04	0.15	0.08	0.04	0.01
Intestines	2.24	0.11	0.73	0.07	0.49	0.04	0.12	<0.01
Bone	65.3	3.15	64.0	2.61	55.8	3.68	47.4	3.17
Muscle	2.47	0.03	2.28	0.03	1.56	0.02	1.00	0.01
Skin	2.13	0.08	0.88	0.03	1.13	0.04	0.36	0.01
Gonads			0.15	0.06				
Tail					3.23	0.53		
Urine	0.38		8.07		4.32		4.50	
Feces	7.40		19.6		26.5		44.4	



TEXT-FIG 1 Proportion of plutonium found in excreta and skeleton of the rat after intramuscular administration of plutonium in +3, +4, and +6 valence states Values corrected for absorption from the left leg



TEXT FIG 2 Loss of plutonium from the hind left leg of the rat after intramuscular injection of the +3, +4, and +6 valence states The ordinate scale gives the per cent of plutonium remaining at the injection site

bone (Fig 1) The picture of plutonium deposition at 8 days was the same as that found after 256 days In other words, no shifting or redistribution of plutonium took place after its initial deposition in the adult animal Fig 2 shows a section in which the periosteum was fortuitously stripped off of the bone The radioautograph demonstrates that a large amount of plutonium is present in this stripped layer

Conclusions

No significant variations in the metabolic behavior of plutonium were observed in each of its three valence states It is probable that plutonium, when absorbed and distributed throughout the body, exists in a single valence state which is independent of its valence at the time of administration

Plutonium is not absorbed from the gastrointestinal tract to any significant degree Following parenteral administration, the chief organ of deposition is the skeleton The soft tissues having the greatest concentration of plutonium are liver, kidney, and spleen, their content of plutonium per gm , however, is from one-fifth to one-tenth that of bone

The chief channel of elimination is the digestive tract The rate of plutonium excretion is very slow and its half period of retention in the body is estimated to be greater than 2 years

Radioautographic studies show that plutonium is deposited primarily in the region of the periosteum, endosteum, and the endosteal covering of the trabecular bone Examination of many of these preparations suggests that the principal site of deposition is on the bone surface and that a very limited accumulation takes place in the mineral structure of the bone No significant redistribution of plutonium took place in the bone during the period of these experiments

The selective localization of the plutonium on the bone surfaces explains the high toxicity of plutonium compared to equivalent quantities of radium This effect arises from the fact that the radium is distributed throughout the mineral portion of the bone, and a large proportion of the α-particles is absorbed before they can enter the marrow cavity Plutonium α-particles can bombard bone marrow more readily, since there is relatively less self-absorption of radiation by the bone

SUMMARY

Detailed metabolic studies of Pu^{239} administered to rats intramuscularly in the +3, +4, and +6 valence state are presented Plutonium was not found to be absorbed from the gastrointestinal tract to any extent The skeleton was the main organ of deposition of plutonium, and the degree of retention in this organ was very great No significant differences were

observed in the metabolic properties of plutonium absorbed by the body for its three valence states Radioautographs demonstrate the deposition of plutonium in the region of the endosteum, periosteum, and the endosteal covering of the trabecular bone

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EXPLANATION OF PLATES

Plate 1

Fig 1 Section of femur from an adult rat injected intramuscularly with 15 γ of plutonium and sacrificed after 8 weeks The radioautograph demonstrates the superficial deposition of plutonium in the region of the periosteum, endosteum, and trabecular bone Hematoxylin, eosin, and silver nitrate, $\times$ 8

Plate 2

Fig 2 Radioautograph and section of femur from a rat injected intramuscularly with 25 γ of plutonium and sacrified after 5 weeks During the preparation of the sections the periosteum became separated from the shaft, and the autograph shows a heavy deposit of plutonium in this stripped layer No calcium was detected in this particular area Hematoxylin, eosin, and silver nitrate, $\times$ 10 4

(Scott Axelrod Fisher Crowley and Hamilton Plutonium)

2

A STUDY OF THE MECHANISM OF "PHOSPHOTRANSFERASE" ACTIVITY BY USE OF RADIOACTIVE PHOSPHORUS*

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"Phosphotransferase" activity, which has been previously described (1), involves the enzymic transfer of phosphate from various aryl phosphates to particular hydroxyl compounds, apparently without the mediation of a nucleotide It was indicated in this work that the phosphate group was transferred directly from the donor to the acceptor compound The main basis for this belief was the observation at the enzyme plus inorganic phosphate did not transfer phosphate to the cceptor compound Nevertheless it remained possible that the accepted phosphate arose from the pool of inorganic phosphate (which is always present since "phosphotransferase" activity is always accompanied by phosphatase activity), and the cleavage of the donor phosphate compound only provided the energy for the esterification

It has now been possible through the use of P^{32} to prove the direct transfer of phosphate from donor to acceptor This has been shown in two ways When unlabeled *p*-nitrophenyl phosphate was used as a phosphate donor in the presence of labeled inorganic phosphate, the synthesized phosphate ester contained virtually no labeled phosphate But when the same donor contained labeled phosphate and was used with unlabeled inorganic phosphate, the synthesized ester did contain labeled phosphate as active as that present in the donor

In summary, the tests were conducted by subjecting *p*-nitrophenyl phosphate to the action of purified citrus phosphatase (which exhibits "phosphotransferase" activity very markedly) in the presence of methyl alcohol and inorganic phosphate After incubation the increase in free nitrophenol was measured, showing the amount of nitrophenyl phosphate split At the same time the increase in inorganic phosphate was measured to determine how much of the phosphate split from the substrate had been reesterified to methyl phosphate, as explained in the earlier paper (1) The methyl phosphate thus formed was isolated from the reaction mixture, and the specific radioactivity of the phosphorus was determined As this value is independent of the amount recovered, considerable loss in yields

* Enzyme Research Laboratory Contribution No 112

was taken in order to insure high purity of the isolated product. Results are compared in terms of specific activity (counts per minute per mg. of P), nevertheless the yields of purified methyl phosphate were large enough to account for about a quarter of the synthesis calculated from the analyses of the digest.

Preparation of Labeled $POCl_3$—The labeled $POCl_3$ was made by a method somewhat similar to that reported by Lindberg (2). A mixture of 6.29 gm. of 85 per cent H_3PO_4 and 1 ml. of a dilute aqueous solution of $Na_2HP^{32}O_4$ was aerated on a hot-plate until the decrease in weight showed that the solution was anhydrous, then thoroughly mixed with 15 gm. of PCl_5, and heated to give a yield of 80 per cent (based on PCl_5) of redistilled $POCl_3$.[1] Starting with 1.1×10^6 counts per minute, 3.13×10^5 counts per minute were recovered, the maximum possible recovery was 3.76×10^5.

Synthesis of Labeled p-Nitrophenyl Phosphate—The procedure previously used for the synthesis of inactive *p*-nitrophenyl phosphate was employed with some modification. To 310 mg. of *p*-nitrophenol, dissolved in 1.4 ml. of dry $CHCl_3$, 1.0 ml. of labeled $POCl_3$ was added, followed by 0.34 ml. of dry pyridine, care being taken to provide good agitation and cooling. After 30 minutes several pieces of ice were added and the reaction mixture allowed to stand several hours to insure decomposition of the acid chlorides. The *p*-nitrophenylphosphoric acid was obtained as a sticky tar by extraction with $CHCl_3$ and removal of the solvent with a stream of air. The crystalline sodium salt of the acid was obtained by adding 2 ml. of H_2O and enough sodium alcoholate to make the solution just alkaline to phenolphthalein, followed by the addition of a large excess of 1:1 acetone and ethanol. The crystals, after washing with 1:1 ethanol-ether, weighed 100 mg. As a further step in purification, the product was converted to the barium salt by dissolving it in 3 ml. of H_2O, adjusting the pH to the pink of phenolphthalein, and adding a slight excess of barium acetate. A slight precipitate was removed by centrifugation, and the supernatant was made 78 per cent with respect to ethanol. The resulting precipitate was washed twice with the same volume of 95 per cent ethanol and finally with ethyl ether. P content, found 7.56 per cent, calculated (for the trihydrate) 7.60 per cent. These crystals were found to be crystallographically identical with barium *p*-nitrophenyl phosphate prepared from an authentic sample of disodium *p*-nitrophenyl phosphate $\cdot 2H_2O$. Approximately 100 mg. of the crystals were diluted with the barium salt derived from 500 mg. of inactive disodium nitrophenyl phosphate for use in the following experiment.

[1] For the most efficient utilization of radioactive material one should use the H_3PO_4 as the limiting starting material, in accord with the stoichiometric proportions of 1 mole of H_3PO_4 to 3 moles of PCl_5 as determined by Geuther (3).

"Phosphotransferase" Reaction with Labeled Nitrophenyl Phosphate—454 mg of the barium salt were converted quantitatively to the sodium salt and mixed with an equivalent quantity of ordinary sodium phosphate Acetic acid was added to pH 5 0, also 9 cc of methanol, 6 cc of M acetate buffer (pH 5 0), and finally enzyme, to a total volume of 62 cc The enzyme employed was 708 units of navel orange juice phosphatase, Preparation D (4) After 6 hours at 38°, tests showed that 91 2 per cent of the original nitrophenyl phosphate had been digested, with the appearance of only 28 6 per cent of the cleaved phosphate in the inorganic form, thus indicating that 22 4 mg (71 4 per cent) of the original phosphorus had been reesterified to methyl phosphate

Methyl phosphate was thereafter isolated from the reaction mixture as $BaMePO_4 \cdot H_2O$, essentially as described in the earlier paper (1) Two consecutive crystallizations from hot water were employed 80 3 mg of barium methyl phosphate (dried *in vacuo* at 56° to constant weight) were recovered

The specific radioactivity of the phosphorus was found to be 29 2 $\pm$ 0 5 counts per minute per mg of P in the initial barium nitrophenyl phosphate and 29 1 $\pm$ 0 5 in the recovered barium methyl phosphate

"Phosphotransferase" Reaction with Radioactive Inorganic Phosphate—An even better proof of the direct transfer of phosphate from the donor to methyl alcohol is shown by the fact that in the presence of relatively large amounts of labeled inorganic phosphate the synthesized methyl phosphate was almost free from labeled phosphate The reaction mixture contained 100 mg of Na_2-*p*-nitrophenyl phosphate dihydrate, 3 ml of methanol, 2 ml of M acetate buffer, pH 5 0, 168 units of *p*-nitrophenyl phosphate phosphatase (lyophilized navel orange juice phosphatase Preparation D), 0 1 ml of labeled phosphate containing 0 678 mg of P and having 5 84 $\times$ 10^5 counts, and water to give a final volume of 21 ml The pH was adjusted to 5 0 with acetic acid Digestion was carried out at 38° for 5 hours, following which the solution was treated in essentially the same manner as before for the isolation of the methyl phosphate In this case, however, after the reaction was stopped, inactive Na_2MePO_4, equivalent to 500 mg of $BaMePO_4 \cdot H_2O$, was added to facilitate the recovery of the enzymically synthesized methyl phosphate, and 910 mg of Na_2HPO_4 were added to dilute the labeled inorganic phosphate and minimize contamination of the methyl phosphate After two crystallizations the specific activity did not change with further recrystallization The final product weighed 121 4 mg after drying over P_2O_5 at 56° *in vacuo* and had a count of 368 per minute Analyses showed that 38 6 mg of nitrophenol had been liberated during the reaction but only 3 71 mg of inorganic phosphorus (equivalent to 43 3 per cent of the total nitrophenyl phosphate cleaved) The esterified

phosphorus corresponded to 41.8 mg. of $BaMePO_4 \cdot H_2O$. Since 500 mg. of that substance had been added as carrier, complete recovery of $BaMePO_4$ should have yielded 541.8 mg. The total count of the $BaMePO_4 \cdot H_2O$ (for the 41.8 mg. of synthesized phosphate ester) was therefore 1640 or a specific activity of 336 counts per minute per mg. of P. Had the phosphorus of the synthesized compound come from (or through) the inorganic pool, its specific activity could not have been less than 6.4×10^4, even in the least favorable (and really impossible) case in which all the phosphate was split from nitrophenyl phosphate before any of the methyl ester was synthesized.

In another experiment carried out under similar conditions the relative specific activity found for the synthesized $BaMePO_4 \cdot H_2O$ was 125 as compared to the hypothetical lower limit of 1.3×10^5. The contribution from the inorganic pool is therefore regarded as negligible.

SUMMARY

By using, in one case, P^{32}-labeled nitrophenyl phosphate, and, in another, radioactive inorganic phosphate it has been shown that in the "phosphotransferase"-catalyzed transfer of phosphate from nitrophenyl phosphate to methanol the transported phosphate does not pass through the inorganic stage.

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STUDIES ON THE PHOSPHORUS METABOLISM OF GREEN ALGAE AND PURPLE BACTERIA IN RELATION TO PHOTOSYNTHESIS

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The concept of circulating phosphate bond energy has been proposed as a basic mechanism for energy transfer in the chemosynthetic and photosynthetic reduction of CO_2 (1-4) Participation of phosphorylated intermediates in the "light" reactions of intact photosynthetic microorganisms has not been established, although several attempts have been made to demonstrate such phenomena (2, 5) To date, experiments with living cells show that a significant reproducible net change in internal phosphorus distribution is generally difficult to establish, even in cases in which the metabolism is known to involve phosphorylated intermediates Hence, it is necessary either to prepare extracts capable of performing partial reactions in which intermediates accumulate or to study phosphorus turnover with a radioactive tracer (P^{32})

The preparation of extracts having the capacity of reducing CO_2 under the influence of light has not yet been successful We have therefore investigated the phosphorus turnover The results obtained in this investigation indicate either that phosphorylation is an integral feature of the photosynthetic process proper or that non-related "dark" phosphorylative reactions are greatly stimulated in some way as a consequence of illumination The existence of a true light-stimulated respiration or fermentation accompanied by gas consumption or production is considered to be very unlikely (6) However, the possibility that other "secondary" phosphorylative reactions not associated with CO_2 reduction are indirectly affected by illumination has not been eliminated conclusively

In the course of the experiments, it has been found that the total phosphate content and its distribution in the cells are strongly dependent on the inorganic phosphate content of the growth medium Cells grown in "high" phosphate media store an appreciable quantity of soluble phosphate which is readily lost when they are suspended in water, saline, and other solutions The removal of a large fraction of this excess phosphate by washing has no apparent effect on the ability to carry on photosynthesis These incidental observations on phosphate nutrition are of importance to the

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problems of photosynthesis only in so far as their cognizance is desirable for intelligent planning of future experiments They are perhaps of more interest as an illustration of a phenomenon which appears to be rather wide-spread among many types of microorganism

EXPERIMENTAL

Preparation of Cultures—Two species of Chlorococcales were used, *Chlorella pyrenoidosa* and *Scenedesmus* D_3 (Gaffron)[1] Both organisms were grown in pure culture in the medium described below (a modification of Gaffron's medium (7)) Part A, tap water 1000 ml , NH_4NO_3 0 52 gm , $MgSO_4 \cdot 7H_2O$ 0 52 gm , and KH_2PO_4 0 208 gm (0 0125 gm for the "low" P medium), Part B, tap water 100 ml , $KHCO_3$ 5 gm , and $NaHCO_3$ 5 gm

Parts A and B were sterilized separately by autoclaving (Part A in bubbling flasks of the Warburg type) 10 ml of Part B were added to each 240 ml of Part A under sterile conditions to furnish the complete growth medium The inoculated flasks were maintained in a water bath at 20–25°, aerated continuously with 5 per cent CO_2-95 per cent air-gas mixture, and illuminated with incandescent lamps Cultures were ordinarily harvested for use 3 to 5 days after inoculation (not longer than 1 day after the density appeared to be maximal) Several experiments were made with cells grown in a medium as above with the addition of $\sim 10^{-5}$ M $FeCl_3$ and trace elements as recommended by Arnon (8) Cells grown in this supplemented medium showed no significant differences in phosphate turnover compared with cultures grown in the usual manner

Rhodospirillum rubrum (strain SI) was grown anaerobically in glass-stoppered bottles with a yeast autolysate-mineral salt medium similar to that described by van Niel (9), with sodium acetate or *n*-butanol as hydrogen donor Cultures were grown in media containing different concentrations of phosphate, these are indicated in connection with the results of the particular experiments The bottle cultures were incubated at $\sim 25°$ under constant illumination with incandescent lamps

Manometric Measurements—Rates of photosynthesis and respiration of the algae were determined by measuring the rate of oxygen exchange in 0 1 M $NaHCO_3$ (*Scenedesmus*) or Warburg's Buffer 9 (*Chlorella*) with air as the gas phase Respiratory measurements were made either by covering the bath with a dark cloth or by wrapping the individual vessels with aluminum foil The foil is very effective in excluding light and was particularly useful in the P^{32} turnover experiments, in which it was desirable to run dark and light samples simultaneously in the same bath Saturation

[1] We are indebted to Dr C B van Niel of Stanford University for supplying the strains of algae and purple bacteria used for these experiments

light intensity was provided by a bank of 60 watt lamps above the vessels The temperature was maintained at 25° or 30°, as indicated It should be noted that rather dense suspensions were used in order to facilitate the P^{31} analyses There must have been considerable "shading" in the light, since dilution of the suspensions usually increased the ratio, O_2 produced in the light-O_2 consumed in the dark

The photosynthetic CO_2 assimilation by *Rhodospirillum rubrum* was followed with bacteria suspended in 0 05 per cent $NaHCO_3$ under an atmosphere of 5 per cent CO_2 in N_2 (10, 11) Sodium butyrate or *n*-butanol served as hydrogen donor Changes in the bicarbonate content of the liquid phase were determined in control vessels by tipping in H_2SO_4 at the beginning and end of the experiment Gas production during the dark "autofermentation" was measured in a similar manner in foil-wrapped vessels

Phosphorus Determinations—Total phosphate was determined as inorganic orthophosphate after digestion of the material with H_2SO_4 and H_2O_2 After clearing the sulfuric digests with several drops of 30 per cent H_2O_2, they were diluted with distilled water and boiled vigorously to hydrolyze pyrophosphate Aside from the use of Pictol (Mallinckrodt, in Na_2SO_3 + $NaHSO_3$ solution) in place of the usual reducing agents, the method of Lohmann and Jendrassik was followed (12) Optical densities were measured in a Klett-Summerson photoelectric colorimeter (Filter 66) after approximately 1 hour's development Inorganic phosphate standards were always included Differences in P^{31} assay of the order of 5 to 10 per cent were not considered significant, even when there appeared to be a "trend" in the data

Inorganic phosphate in trichloroacetic acid (TCA) extracts was estimated by the Fiske-Subbarow method (13) with Pictol as the reducing agent

Tests for labile phosphate esters were made by the procedure recently developed by Lowry and Lopez (14) With ascorbic acid as the reducing agent in acetate buffer solution at pH 4, presumably only true inorganic phosphate is detected by this procedure Differences between the values obtained by this method and those shown by the Fiske-Subbarow method are ascribed to labile esters (acyl phosphate, etc)

Radioactivity Measurements—Aliquots of the sulfuric acid digests were also used for assay of P^{32} All samples (saline washes, etc) were neutralized to the phenolphthalein end-point and evaporated to dryness on small watch-glasses under infra-red lamps Activity was determined by means of a conventional end-window Geiger-Müller counting tube connected with a scale-of-64 circuit Corrections for decay or self-absorption were unnecessary In all experiments radioactivity balances were obtained and recoveries of labeled phosphate summed up over all chemical fractions

as compared with the initial labeled content of the whole cells were always greater than 90 per cent.

Fractionation of Cells—Thus far only crude fractionations have been made, primarily because of difficulties which are discussed below. In the "uptake" experiments, bacteria or algae were exposed to solutions containing P^{32} (2 to 5 microcuries per ml.) in the form of inorganic phosphate under the same conditions as for manometric measurements. Each vessel usually contained ~0.1 ml. (wet volume) of cells. In a number of experiments with the algae small Erlenmeyer flasks (25 ml.) were used rather than Warburg vessels. In these instances, control manometric measurements were simultaneously made with aliquots of the algal suspension in order to be sure that the cells were photosynthesizing and respiring normally.

After suitable intervals of time, the suspensions were extracted directly or the cells washed and then extracted with ~10 ml. of cold TCA (final concentration 5 to 6 per cent) for about 1 hour at 5°. For the usual uptake experiment, the organisms were harvested and used without washing, the TCA extract was discarded, and the residue washed twice with 10 ml. portions of cold 5 per cent TCA. Specific activity (counts per minute per microgram of P^{31}) in the gross residue was then determined.

Fractionation of the TCA-insoluble residue was accomplished by a procedure developed for analysis of the same fraction of yeast.[2] As applied here, this consisted of the following steps. Lipides were removed with cold alcohol and subsequent repeated extractions with hot ether-alcohol mixture (3:1). The residue from the lipide extraction was then treated with 1 N KOH for 24 hours at 37°. This treatment brings phosphoproteins and nucleic acid phosphate into solution. Specific activities in the lipide fraction, KOH extract, and KOH residue were determined in the usual manner.

When the cells could be washed after exposure to P^{32}, the TCA extracts were retained for specific activity determinations (in these instances, the residue was washed only once with several ml. of 5 per cent TCA and the wash added to the extract).

Results

Phosphorus Exchange between Cells and Medium—Resting cell suspensions are ordinarily prepared by harvesting a culture and washing the cells thoroughly in order to remove traces of nutrients and other substances remaining from the occluded culture fluid. The washing liquid is usually distilled water, 0.85 per cent saline, or less frequently fresh culture medium. It is well known that substances must be readily lost from bacterial cells,

[2] Juni, E., Kamen, M. D., Reiner, J. M., and Spiegelman, S., in press.

since washing frequently leads to "inactivation" of the organisms (15) In spite of this possibility, washing is almost invariably necessary in P^{32} uptake experiments, since an excess of the tracer generally remains in the external medium Consequently, the effect of washing on the internal phosphate composition was studied Table I illustrates typical data obtained when organisms grown in the presence of P^{32} are washed with saline

TABLE I

Leakage of P^{32} from Internally Labeled Cells of Chlorella pyrenoidosa and Rhodospirillum rubrum (SI) into Successive Washes of Saline and Growth Medium at 0°

The results are expressed in counts per minute

Experiment A, 5 day culture, Experiment B, 4 day culture, Experiment C, 5 day culture, Experiment D, 8 day culture The washes consisted of 25 ml portions of ice cold solution Centrifugations were made in a refrigerated centrifuge at 0° as rapidly as possible In all cases, the saline was 0 85 per cent For *Chlorella*, the medium was Part A of the growth medium (approximately 50 γ of P per ml) For *Rhodospirillum*, the medium was the same as the complete growth medium (700 to 800 γ of P per ml) In Experiment B, approximately 0 3 ml wet volume of algae was washed in each case In Experiment C, 87 mg , dry weight, of bacteria were washed in each series

	Chlorella pyrenoidosa				*Rhodospirillum rubrum* (SI)		
	Experiment A		Experiment B		Experiment C		Experiment D
	Saline	Medium	Saline	Medium	Saline	Medium	Medium
Wash 1	625,000	326,000	1,040,000	800,000	382,000	117,000	330,000
" 2	401,000	179,000	343,000	425,000	117,000	8,350	40,600
" 3	162,500	140,000	35,400	77,500	36,000	5,130	17,100
" 4	58,500	88,000	21,000	32,000	12,800	5,650	15,000
" 5	22,800	80,500	16,100	16,000	1,800	5,450	10,200
Washed organisms	2,420,000	2,850,000	1,847,500	1,806,000	374,000	683,000	1,125,000
Original supernatant per ml	238,800	238,800	410,000	410,000	232,000	232,000	166,000

and phosphate-containing solutions The phosphate in all fractions of such cells is necessarily labeled and completely equilibrated with exogenous phosphate, *i e*, external and internal phosphate have the same specific activity

From these data, it is evident that internal phosphate is gradually removed by successive washes under conditions in which metabolism is presumably at a low level (0°) Similar results were found in experiments with *Scenedesmus* D_3

The effects of washing on P^{31} distribution in *Rhodospirillum rubrum* are shown in Table II The data were obtained by suspending the washed bacteria from Experiment C of Table I in 15 ml of saline and extracting 1 ml aliquots with TCA Equal quantities of cells were used in each series (87 mg, dry weight)

If the specific activity in the external medium and the total number of counts associated with the cells originally (washes plus washed cells) are known, it is possible to make a fairly accurate estimate of the amount of P^{31} initially present in the bacteria (about 177 γ per ml) Further, if we assume that the acid-insoluble phosphate does not exchange with the medium, as appears to be the case from the specific activities given in Table II, then it is clear that saline washes removed ~87 per cent of the phosphate initially in the soluble fraction, whereas washing with medium

TABLE II

Distribution of P^{31} and P^{32} in Rhodospirillum rubrum (SI) after Washing with Saline and Medium

	Saline washed		Medium washed	
	γ	*counts per min per γ*	γ	*counts per min per γ*
Total P*	75 7	329 0	321 5†	141 7
P in TCA extract	15 3	326 0	254 5	108 5
" " " residue	60 4	335 0	51 8	345 0

Specific activity in original supernatant ≅ 356 counts per minute per microgram (651 γ of P^{31} per ml)

* Direct determinations, per ml of suspension

† Of the 321 5 γ, 81 γ of specific activity 6 5 were *not* associated with the cells

increased the phosphate content of this portion by 49 per cent The increase in phosphate content in the latter case is a *net* increase, since a considerable amount of the original phosphate was lost as shown by appearance of P^{32} in the washes Similar experiments with *Scenedesmus* D_3 and *Chlorella pyrenoidosa* showed the same phenomenon, *i e*, cells grown in P^{32} can be differentially labeled simply by washing with unlabeled phosphate-containing solutions (room temperature)

Algae which have been washed with saline still show apparently normal photosynthetic and respiratory activity in spite of considerable loss of phosphate This suggested that the "excess" soluble phosphate is dispensable to the cell It was found that this storage can be eliminated by growing the algae in a medium containing very little inorganic phosphate (see the "low" phosphate medium under "Preparation of cultures") In Table III, the effects of washing on the internal P^{31} distribution in *Chlorella pyrenoidosa* grown in "high" and "low" phosphate media are compared

Analyses for orthophosphate in the TCA extracts of Experiment 2 of Table III showed that the loss into the washes occurs at the expense of the TCA-soluble inorganic phosphate With the Lowry-Lopez procedure (14), no labile phosphate esters of the acyl phosphate type could be detected in extracts of organisms grown in either type of medium It is of interest that the final concentration of orthophosphate in the medium of full grown cultures on "high" phosphate is about 40 γ per ml, while the "low" phosphate medium is usually entirely depleted of phosphate This difference parallels that observed by Mann (16) in growing cultures of *Aspergillus niger* This mold grown in 0 2 to 0 5 per cent K_2HPO_4 media

TABLE III

P^{31} Distribution in Chlorella pyrenoidosa Grown in "Low" and "High" Phosphate Media

All cultures were 5 days old The results are expressed in micrograms

Medium Experiment No Treatment	Low phosphate 1a Not washed	Low phosphate 1b Washed*	High phosphate 2a Not washed	High phosphate 2b Washed*
P in TCA extract	41 2	32 0	149 0	90 3
" " " residue	183 0	191 0	404 0	374 0
" " Wash 1		0		16 8
" " " 2				17 2
" " " 3				10 7
Total P	224 2	223 0	553 0	509 0

* The algae were washed with three 10 ml portions of water at room temperature (approximately 0 2 ml of wet volume algae in each series)

contains 1 to 2 per cent of its dry weight as phosphorus in contrast to 0 3 per cent when the medium contains only 0 02 per cent K_2HPO_4 In the latter case, the medium is depleted of phosphate rapidly Mann has also demonstrated significant disparities in the metabolism of the two types of cultures

P^{32} Uptake—Typical results showing the effect of light on P^{32} uptake by *Chlorella* and *Scenedesmus* are given in Fig 1, where specific activity in the TCA-insoluble residue is plotted as a function of time Each point represents the average of duplicate determinations In all of the experiments performed, there were no significant or consistent differences between light and dark samples with respect to P^{31} content of any of the fractions examined The acid-soluble portion has as yet, however, not been examined in detail

The turnover involving the insoluble fraction is distinctly greater in the light than in the dark Moreover, the extent of this turnover is not di-

rectly proportional to the metabolic activity as indicated by gas exchange For example, in the experiment of Fig 1, *B*, the rates of O_2 absorption and production observed in control vessels were as follows (*a*) In the ab-

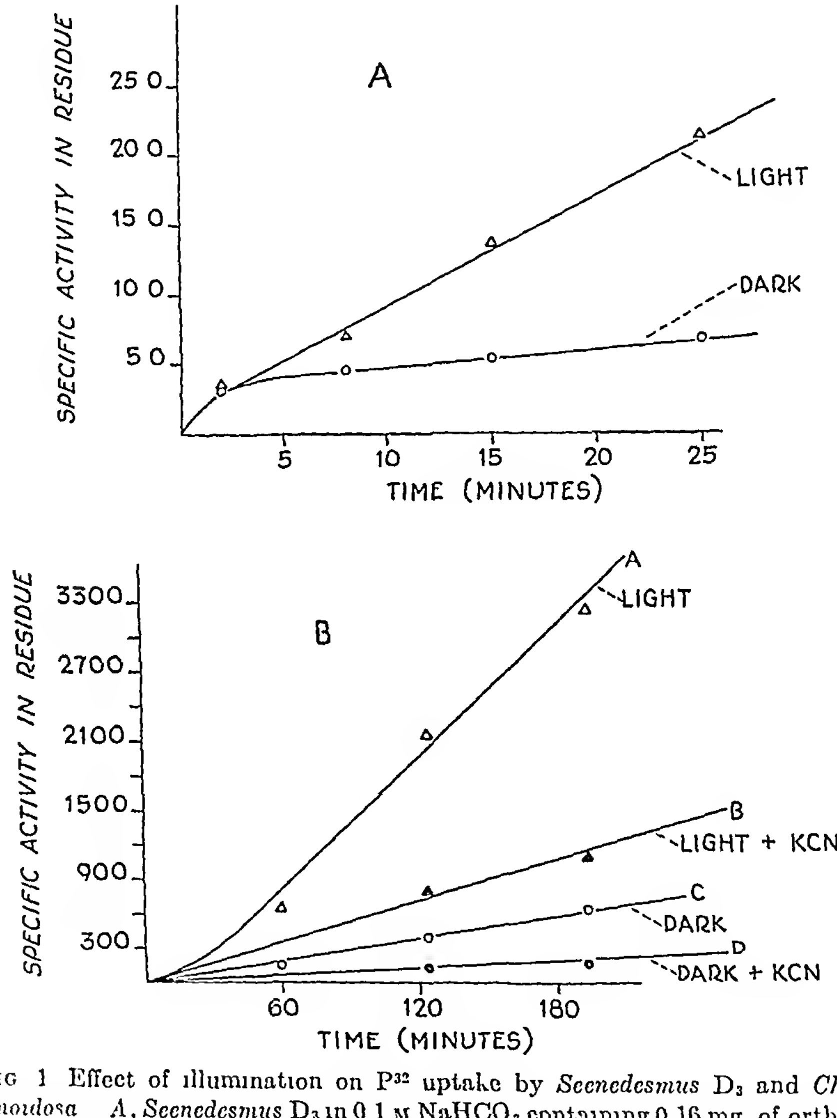

FIG 1 Effect of illumination on P^{32} uptake by *Scenedesmus* D_3 and *Chlorella pyrenoidosa* *A*, *Scenedesmus* D_3 in 0 1 M $NaHCO_3$ containing 0 16 mg of orthophosphate as P per ml and 0 76 mg of $(NH_4)_2SO_4$ per ml, temperature, 30° *B*, *Chlorella pyrenoidosa* in Warburg Buffer 9, temperature, 22°, final concentration of KCN, $4\ 55 \times 10^{-3}$ M

sence of KCN, photosynthesis (corrected for respiration) = +129 microliters of O_2 per 30 minutes, respiration = −36 2 microliters of O_2 per 30 minutes (*b*) In the presence of KCN, photosynthesis (corrected) = +37 microliters of O_2 per 30 minutes, respiration = −43 5 microliters of O_2 per 30 minutes

Thus, although the endogenous respiration (dark) is stimulated slightly by KCN, the incorporation of P^{32} into the residue is markedly less in the presence of KCN than in its absence (Fig 1, *B*, Curves C and D) The lack of correspondence between gas exchange and phosphate turnover can also be seen by comparing Curves B and D with the manometric data given for Fig 1, *B* This indicates that the differences observed cannot be attributed simply to a greater general metabolic activity during illumination

Results of other uptake experiments with *Chlorella pyrenoidosa* are listed in Table IV

The effect of illumination on phosphate turnover into the insoluble fraction in *Rhodospirillum rubrum* is more pronounced than that observed in the green algae In the experiments summarized in Table V, the amount of CO_2 liberated by "autofermentation" in the dark was of the same order of magnitude as the CO_2 assimilated in the light (This was true in Experiment B of Table V also, even though an attempt was made to deplete the endogenous reserves by aerating the cells in 0 05 per cent $NaHCO_3$ for 1 75 hours in the dark before use) Considered with the P^{32} results, this again denotes the lack of a strict relation between extent of phosphate turnover and the over-all metabolic level as indicated by gas exchange A large differential in the specific activity is seen even in the endogenous controls (Table V, Experiment B, Column *a*), apparently there was sufficient hydrogen donor present in the cells to permit extensive photosynthesis ("autoassimilation")

Delineation of the rôle played by phosphorylation in CO_2 reduction will depend to a great extent on a knowledge of the compounds actively engaged in the turnover of phosphate between the soluble reservoir and other portions of the cell A preliminary attempt to identify the nature of these compounds was made with aliquots of the same bacterial suspension that was used for Experiment B in Table V Results of a crude fractionation of the cells are presented in Table VI After 100 minute exposures to light and dark, the cell suspensions were removed from the Warburg vessels, centrifuged, and the cells washed three times with 15 ml portions of cold 0 85 per cent saline Fractionations were then performed as described in the experimental section

Recent work indicates that orthophosphate enters the "soluble" portion of the cell probably as a phosphoester and that organic phosphate compounds are subsequently derived from a reservoir in this fraction (17) This appears to be also true for photosynthetic microorganisms as evidenced by the fact that the highest degree of equilibration with exogenous phosphate is found in the soluble fraction (Tables IV and VI) Ordinarily, the extent of equilibration is easily determined in P^{32} uptake experiments,

in those reported here, the estimates are complicated by the phosphate exchange occurring between cells and medium For example, in the experi-

TABLE IV

P^{32} Uptake by Chlorella pyrenoidosa in Warburg Buffer 9

Experiment A 5 day-old culture grown in "high" phosphate medium, temperature, 20°

Time	Fraction	Light: Counts per min per γ P^{31}	Light: Per cent equilibration*	Dark: Counts per min per γ P^{31}	Dark: Per cent equilibration*
min					
118	Whole cells†	1560	2 36	524	0 76
		1825		560	
	TCA soluble phosphate (calculated)	6320	8 8	2120	2 96
	TCA residue	406	0 57	131	0 17
		404		111	
273	" "	2120	2 88	368	0 51
		2000			

Experiment B 4 day old cultures grown in "low" and "high" phosphate media, duration of experiment, 165 min, temperature, 25°

	Origin of culture			
	High phosphate		Low phosphate	
	Light	Dark	Light	Dark
P^{32} in TCA residue, *counts per min per γ*	298 0	89 0	2490 0	885 0
	266 0	106 0	2320 0	850 0
P^{31} in residue, γ	50 3	50 5	41 0	36 6
	52 0	53 8	41 9	41 7
Total P^{31}, γ	242 6‡		63 4	
O_2 per 30 min, *microliters*	+163 4§	−28 0	+158 6§	−20 1

$$* \frac{\text{Counts per min per microgram } P^{31}}{\text{Counts per min per microgram } P^{31} \text{ in external medium } (= 71{,}700)} \times 100$$

† Cells washed three times with 10 ml portions of water before measurement

‡ 71 γ were found to be in the suspension liquid as orthophosphate (only 2 2 γ in the low phosphate experiment)

§ Corrected for respiration

ments with *Chlorella pyrenoidosa* summarized in Table IV, Experiment A, the external specific activity at the end of the experiment was 71,700 counts per minute per microgram of P^{31}, while the specific activity of the added

tracer was 1,120,000 Analyses for P^{31} showed that the external orthophosphate, initially from the tracer alone, had been diluted with unlabeled orthophosphate released from the cells In this particular case, the final external specific activities were identical in light and dark samples

The extent of equilibration in the various fractions of *Rhodospirillum rubrum* (Table VI) is more difficult to determine Although the culture was grown in a medium with only a moderate concentration of inorganic phosphate (~40 γ of P per ml), a rather large amount of phosphate was released into the solution while the culture was being aerated prior to the

TABLE V

P^{32} Uptake by Rhodospirillum rubrum (SI) in 0 05 Per Cent $NaHCO_3$

Experiment A, 7 day culture grown in "high" phosphate medium (700 to 800 γ of P per ml) with *n* butanol as H donor *n*-Butanol was used also as H donor for the uptake experiment Gas phase, 5 per cent CO_2 in N , temperature 25°, duration of experiment, 230 minutes, total P^{31}, 297 γ

Experiment B, 68 hour culture grown in "low" phosphate medium (~40 γ of P per ml) with acetate as H donor Sodium butyrate was used as the H donor for the experiment In Column *a*, light and dark, no H donor was added Gas phase, 5 per cent CO_2 in N_2, temperature, 30°, duration of experiment, 150 minutes (cells "dissimilated" by aeration prior to experiment), total P, 117 γ

	Experiment A					Experiment B					
	Light		Dark			Light			Dark		
	(a)	(b)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)
P^{32} in residue, *counts per min per γ*	159	175	8	8	7	2310	3070	3110	356	299	276
P^{31} in residue, γ	154	142	141	148	145	94	91	96	88	90	88

experiment proper (767 γ per 40 ml , after resuspension in 40 ml of fresh 0 05 per cent $NaHCO_3$, 5 ml aliquots of the suspension were used for the experiments reported in Table VI) The tracer added had a specific activity of about 66,000 counts per minute per microgram of P^{31} Judging from the final values of the specific activities in the external medium (P^{32} supernatant liquid), even more internal phosphate was released during the course of the experiment The activities observed in the washes represent very little P^{31} (<2 γ per 15 ml in the first wash) If the per cent equilibration is calculated on the basis of the final specific activities in the supernatant liquid as 100 per cent, then the effect of light on the turnover is even more pronounced than is indicated by the ratios of the specific activities given in the last column of Table VI (light *versus* dark)

At any rate, it may be concluded that the uptake and turnover are both much greater in the light than in the dark Although the residue contained

a large quantity of lipide phosphorus, this fraction showed very little turnover. The bulk of the difference is encountered in the KOH extract, which represents "total protein" phosphorus. In contrast to yeast (17),[2] the KOH residue contains no detectable P^{31}, the counts observed in this fraction may well be due to contamination from the KOH extract.

"Flow" Experiments—Interpretation of P^{32} uptake experiments is sometimes complicated by the fact that high specific activity tracers are employed. Under these conditions, rather large amounts of radioactivity

TABLE VI

P^{32} Uptake and Distribution in Rhodospirillum rubrum (SI) during Photosynthesis and Autofermentation

Culture and conditions similar to those described for Experiment B, Table V

	Light			Dark			
	Counts per min (1)	P^{31} (2)	Counts per min per γ P^{31} (3)	Counts per min (4)	P^{31} (5)	Counts per min per γ P^{31} (6)	$\frac{(3)}{(6)}$
		γ			γ		
P^{32} supernatant	374,000	22.9	16,300	2,120,000	45.8	46,250	
Wash 1	27,200	0		86,300	0		
" 2	8,090	0		8,000	0		
" 3	7,500			5,880			
TCA extract	550,500	62.5	8,825	284,000	65.5	4,335	2.04
" residue							
Lipide fraction	24,600	125.4	196	8,900	125.1	71.3	2.75
KOH extract	1,355,000	295.5	4,585	102,025	239.5	353.0	13.00
" residue	1,860	0		375	0		
Total	2,348,750	506.3		2,615,480	525.9		

are associated with quantities of P^{31} which are beyond the range of detection by the usual colorimetric procedures. Appearance of activity in the soluble and insoluble fractions, without a significant change in distribution of P^{31}, does not then necessarily mean that there is any turnover between these fractions, *i.e.*, independent routes may exist for the uptake of minute amounts of P^{31} into the various fractions of the cell. In experiments of this nature, it is desirable to test this possibility by means of the "flow" experiment.

This may be conducted in one of two ways. First, the cells can be grown in the presence of P^{32} and then differentially labeled by suitable procedures. For example, with P^{32}-labeled yeast, fermentation of glucose in the presence of unlabeled phosphate causes a considerably greater diminution of the specific activity in the acid-soluble portion compared

with the acid-insoluble fraction (18) With such yeast, the effect of any given conditions on the flow *between the two fractions* may be readily determined With green algae or *Rhodospirillum rubrum*, the differential in specific activities can be established by the simple expedient of washing with solutions containing unlabeled phosphate as already described This first method, however, is unsatisfactory with photosynthetic microorganisms because the amount of phosphate involved in the light-accelerated turnover is too small to affect the initial specific activities measurably (particularly, if the initial differential is not very great) It would be possible to observe a significant difference in at least one of the fractions if the P^{31} distribution were very disproportionate

In the second method, unlabeled cells are exposed to P^{32} (30 to 50 microcuries per ml of suspension) for a relatively short time and then washed thoroughly to remove occluded tracer This procedure usually results in a relatively high specific activity in the soluble fraction, while that in the insoluble is quite low The cells are then subjected to light and dark in a solution preferably containing no exogenous phosphate Flow between the two cellular fractions can then be followed in a system uncomplicated by exchanges occurring between cells and medium The results of three flow experiments performed by the second method are given in Table VII (in Experiment C, exogenous phosphate was present as indicated in the foot-note, all values are the averages of duplicate or triplicate determinations)

The specific activity in the soluble fraction is invariably higher than that in the insoluble With shorter exposure times (Experiments B and C, Table VII), the initial differential between the two fractions is somewhat enhanced[3] Under the influence of light, the specific activity in the extract fraction decreases significantly more than in the corresponding dark samples This indicates that light stimulated the flow of low specific activity phosphate from the insoluble fraction into the soluble portion The simultaneous flow in the reverse direction is evidenced by corresponding increases in the specific activity of the residue

In Experiment C, Table VII, the stimulatory effect of light on the phosphate turnover is apparent from the specific activities observed in the residues The extract values, on the other hand, show no corresponding changes This seeming discrepancy is explained by the fact that the P^{32} activity in the extract was much greater than that in the residue at the

[3] Similar results are obtained when carbon dioxide fixation is studied with C^{14} as tracer (Allen, M B , and Kamen, M D , unpublished experiments) A discussion of the turnover in the carbon compounds relative to solubility is given in a forthcoming monograph of the Photosynthesis Symposium of the American Association for the Advancement of Science, December, 1947

start of the experiment. Since ~100 γ of the extract phosphorus were due to exogenous unlabeled phosphate, it is clear that the true specific activity of the soluble phosphate in the algae was considerably greater than 2200. Thus, the flow of several micrograms of soluble phosphate into the residue could change the specific activity of the latter 100 per cent, while an identical flow of residue phosphate into the soluble fraction would

TABLE VII

Flow of P between Soluble and Insoluble Fractions in Chlorella pyrenoidosa

Experiment A, 3 day culture grown in "high" phosphate medium. Exposed to P^{32} in Warburg Buffer 9 under good illumination for 20 minutes at 21°. Washed cells resuspended in Warburg Buffer 9 at 23° for 120 minutes.

Experiment B, 2 day culture grown in "low" phosphate medium. Exposed to P^{32} in Warburg Buffer 9 in light for 1 minute at room temperature. Washed cells resuspended in Warburg Buffer 9 at 25° for 197 minutes.

Experiment C, 4 day culture grown in "high" phosphate medium (containing added Fe and trace elements). Exposed to P^{32} in complete medium in light for 2 minutes at room temperature. Washed cells resuspended in complete medium at 20° for times indicated. Approximately 100 γ of the quantities listed as TCA-extractable phosphate in this experiment were present as exogenous phosphate.

Experiment		TCA extract		TCA residue	
		Counts per min per γ P^{31}	P^{31}	Counts per min per γ P^{31}	P^{31}
			γ		γ
A	Zero time	9985	19.0	1705	68.1
	Dark	7810	17.3	2240	67.5
	Light	6890	17.3	2565	60.3
B	Zero time	1970	27.5	113	124.5
	Dark	1073	30.4	280	117.0
	Light	734	34.3	340	115.3
C	Zero time	2175	131.0	159	84.3
	Dark, 80 min	2245	128.5	189	84.1
	" 150 "	2200	132.0	196	91.0
	Light, 80 min	2290	124.8	237	85.5
	" 150 "	2055	127.0	297	84.5

change the specific activity of the extract phosphate by only a few per cent or less.

Estimates of the amount of turnover occurring can be made from these data by calculations involving a number of assumptions. Such calculations indicate that a fairly large fraction of the soluble phosphorus is participating in the light-stimulated turnover. Since the compounds concerned are as yet unknown, the estimates are at best very rough approximations. As an illustration, simple isotope dilution calculations can be applied to Experiment A (Table VII) as follows.

Let a = the specific activity in the residue at zero time, b = the specific activity in the residue at the end of the experiment, x = the quantity of phosphate transferred (this is assumed to be the same in both directions), and 68 = the micrograms of P^{31} in the residue Then, for the dark, b/a = 1 32 Also,

$$\frac{b}{a} = 1\ 32 = \frac{9985x + (68 - x)(1705)}{68\ (1705)}$$

From this equation, $x = 4\ 4\ \gamma$ Solving the analogous equation for the amount moved in the light gives $x = 7\ 1\ \gamma$ Similar calculations in which the data for the soluble fraction are used give about the same result In connection with these estimates, one point should be emphasized here, *viz*, that the ratio of final specific activities in a particular fraction after incubation in light and dark ((specific activity in the light)/(specific activity in the dark)) is not directly proportional to the difference in turnover It may be remarked that a small fraction of the total phosphate of the cells is involved in turnover as evidenced by the low equilibration values and the flow calculations This fact makes the interpretation of simple uptake experiments difficult, particularly in view of the large amounts of phosphate moved about by washing procedures, the results of which in turn are influenced by culture conditions

Finally it is worthy of note that the data of Table IV, Experiment B, and Tables V and VII show no obvious fundamental differences in phosphate turnover between cells grown in "low" or "high" phosphate media

DISCUSSION

Before discussing the implications of these experiments to photosynthetic mechanisms, it seems worth while to consider briefly phosphate nutrition and its effects on the physiology of microorganisms The nutritional aspects of phosphate metabolism have not been systematically investigated, and our information is limited to incidental observations made during the course of experiments dealing with biochemical transformations This is generally true for observations on nitrogen nutrition also The results of Mann (16) with *Aspergillus niger* show that the concentration of phosphate used in the growth medium has a profound effect on (*a*) the rate of phosphate utilization, (*b*) phosphate content, (*c*) rapidity and extent of growth, (*d*) respiratory quotient, (*e*) nitrogen metabolism, (*f*) content of certain vitamins, and (*g*) glucose metabolism Similarly, it has been found (19) that the inorganic nitrogen content of the medium influences the protein content and the enzymatic constitution of *Bacterium coli* to a great extent

In photosynthetic microorganisms, the phosphate content (and dis-

tribution) also depends on the concentration of inorganic phosphate in the medium With "high" phosphate concentration, excess phosphate appears to be stored, primarily in the TCA-extractable fraction This phosphate seems to be dispensable to the cells and readily leaks out into water, saline, etc , even at low temperature Numerous examples of phosphate leakage from cells to medium can be found in data reported for various types of bacteria In *Staphylococcus aureus* and *Streptococcus hemolyticus*, both N and phosphate are released into the supernatant in appreciable quantity compared with the amount extractable with 5 per cent TCA (even at low temperature (20)) Relatively large amounts of both inorganic and organic phosphate are soon found in the supernatants when *Streptococcus faecalis* is suspended in saline in the absence of glucose (21) During endogenous respiration, resting cells of *Thiobacillus thiooxidans* release inorganic phosphate into the medium (4, 22) Leakage of phosphate explains the failure of thoroughly washed *Bacterium coli* to ferment glucose, since addition of inorganic phosphate restores the activity (15) The actual leakage from *Bacterium coli* is evident in the data given by Macfarlane (23) In this case, a young culture of the bacteria after two washings with saline showed a rapid release of inorganic phosphate (into saline) in the absence of glucose An appreciable quantity of the original phosphate of *Trypanosoma equiperdum* is lost when the organisms are washed with saline (24)

Release of phosphate from yeast is reported to be almost negligible The experiments of Hevesy and Zerahn (25) with yeast grown in P^{32} disclosed that only 1 to 2 per cent of the total P^{32} content is lost into inactive nutrient solution (at 20° over a period of 24 hours) Before performing the experiment, however, the yeast was washed thoroughly with nutrient medium (containing phosphate) to remove adhering radioactivity It is possible that some leakage or exchange occurred during the washing According to Reiner,[4] about 10 per cent of the total P^{31} of yeast is released into distilled water over a period of several hours at room temperature (after being washed twice with cold distilled water) It is interesting to note that other experiments of Hevesy and Zerahn (25) showed that yeast internally labeled with K^{42} loses a large fraction of its K into the medium

Prior to the present work it does not appear that any success has attended efforts to demonstrate an unequivocal relation between illumination and phosphate esterification (2, 5, 6, 26) Emerson *et al* (2) attempted to observe shifts in phosphate distribution in various fractions of *Chlorella pyrenoidosa* which might have been expected to result when cells were illuminated in the presence and absence of CO_2 They concluded that, with the possible exception of "Ba-insoluble resistant phosphate,"

[4] Reiner, J M , private communication

no significant changes occurred which could be interpreted as supporting hypotheses that conversion of radiant to chemical energy was mediated by "high energy" phosphate bond formation However, only the TCA-soluble phosphate was fractionated in these researches Our data indicate that TCA-soluble phosphate composition is strongly dependent on culture and washing conditions and procedures and thus it is difficult to assess the relevance of the distributions in P^{31} reported by Emerson *et al* An attempt to use simple uptake of P^{32} as a definitive test for the existence of light coupling to phosphate esterification has recently been reported by Aronoff and Calvin (5) No light stimulation of phosphate pick-up was observed It is evident, however, from our previous discussion of complications in the interpretation of simple uptake experiments and from the effects noted in our researches that the demonstration of phosphorylative mechanisms cannot be established without close attention to effects attendant on non-equilibration, washing procedures, and previous history of the cultures One other point is worthy of comment It has been found that in intact yeast fermenting glucose in the presence of fluoride no acyl phosphate can be detected [5] Hence even under conditions in which a conversion of inorganic to acyl phosphate is reasonably certain to be occurring during metabolism in intact cells, it may be impossible with present analytical methods to demonstrate such phosphorylation

Arguments which have been presented against the notion of a phosphorylation mediated "directly" by light are based on the absence of any photochemical model reactions in which there occurs an efficient parceling of the energy of a single quantum into many smaller quanta 6, 26) [6] It should be noted that this type of objection can be used against *any* theory involving production and storage of energy in energy-rich bonds

It is unnecessary to dwell upon the analogies which have been made between photosynthetic and chemosynthetic CO_2 reduction (27, 28) These may be paraphrased by the statement that mechanisms for CO_2 fixation are similar in both types of metabolism, but the ultimate energy source is different Some evidence exists from the experiments of Vogler and coworkers (4, 27) that there may be mediation of phosphate bond formation by sulfur oxidation uncoupled from CO_2 uptake in *Thiobacillus thiooxidans* The results obtained depended on the determination of inorganic phosphate in the medium during alternate periods of oxidation and CO_2 uptake The assumption required was that disappearance of phosphate from the medium was synonymous with the formation of phosphate esters It appears from our washing data that this assumption may well require more experimental justification Further, an unexplained

[5] Spiegelman, S , private communication

[6] Franck, J , private communication

release of phosphate during endogenous metabolism of the organisms used by Vogler *et al* complicates interpretation of the conclusions reached Other difficulties, such as a very high ratio of CO_2 "fixed" to phosphate "esterified," uncertainty as to the state of reduction of the CO_2 taken up, etc (6), would seem to indicate the desirability for additional experimental evidence bearing on the relation of phosphate esterification to CO_2 reduction It is of interest to record the recent observations of McElroy (29) who has found that extracts from firefly lanterns can be prepared which in the presence of oxygen exhibit an increase in bioluminescence upon the addition of adenosine triphosphate In this process, one observes a release of light quanta concomitant with oxidation, analogous to a "reverse photosynthesis" It appears that a phosphorylative mechanism is involved in absorption of oxygen and production of light However, no evidence exists at present that this effect of "high energy" phosphate is direct

The light-induced phosphate turnover may be useful in monitoring activity of extracts prepared from photosynthetic organisms It may be supposed that extracts which simulate the behavior of intact cells with respect to stimulation of phosphate turnover by light may be good test systems for analysis of components required in synthetic mechanisms

SUMMARY

1 The uptake and turnover of phosphate in various cell fractions as influenced by light has been investigated by the use of two species of Chlorococcales (*Chlorella pyrenoidosa*, *Scenedesmus* D_3) and a species of Athiorhodaceae (*Rhodospirillum rubrum*)

2 It is shown that the gross phosphate distribution is altered appreciably by experimental conditions (culture media, washing procedures) prior to analysis of the organisms In particular it is found that organisms grown in media with the phosphate composition usually recommended for optimal growth contain excess inorganic or highly labile phosphate which is easily removed by washing, while organisms grown in media of low phosphate content do not contain such washable phosphate The uncertainty introduced into simple uptake experiments by these phenomena is discussed

3 The phosphate uptake in the light is considerably greater than in the dark for all the organisms examined Because of the uncertainties associated with the trichloroacetic acid-soluble phosphate, uptake data are usually referred to TCA-insoluble phosphate The manometric data obtained with algae with KCN as an inhibitor of photosynthesis show that the uptake of phosphate is not directly related to the respiratory activity as measured by CO_2 evolution In general no rigid proportionality appears to exist between phosphate uptake and the over-all metabolic level

4 The present studies, in which internally labeled organisms are em-

ployed, indicate that cellular phosphate turnover and equilibration with exogenous phosphate are mediated by a small fraction of the soluble cellular phosphate In experiments on flow of phosphate between insoluble and soluble cell fractions, it is found that light stimulates phosphate turnover

5 The results obtained are discussed as well as those from other studies in the literature It appears from these studies that ester phosphate may be formed as a result of light absorption, but that there is no experimental evidence which can decide whether such an esterification is, or is not, directly coupled with light absorption

Addendum—A detailed description of the experiments of Aronoff and Calvin has appeared recently (*Plant Physiol*, 23, 351 (1948)) These workers used an indirect method for determination of inorganic and organic fractions of phosphate (P^{32}) taken up Apparently the P^{31} contents of these fractions were not measured in any instance and consequently no data on specific activities are available Since P^{31} contents were assumed from values given in the literature by other workers using different algae, the significance of the calculations of Aronoff and Calvin relating phosphate uptake and turnover to light and dark metabolism cannot be readily assessed

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HEMOGLOBIN SYNTHESIS FROM GLYCINE LABELED WITH RADIOACTIVE CARBON IN ITS α-CARBON ATOM*

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Important progress in our knowledge concerning the biological precursors of hemin was made when Bloch and Rittenberg (1) presented evidence that significant amounts of deuterium are incorporated into the hemin of rats fed deuterioacetic acid Further advances in this field were made by Shemin and Rittenberg (2–4), who showed that glycine labeled with N^{15} is utilized as a nitrogenous precursor of hemin, whereas other possible amino acids, such as glutamic acid, proline, and leucine, do not act in a similar fashion and contribute only in an indirect way to hemin N by enriching the N^{15} concentration of the body It has been reported recently that hemin synthesis from glycine occurs *in vitro* in the presence of avian blood (5) and with blood from patients with sickle cell anemia (6) It is of considerable interest for an understanding of the mechanism of hemin synthesis to determine whether the α-carbon atom of glycine is also incorporated in hemin

Experiments demonstrating the participation of the α-carbon atom of glycine in the synthesis of hemin are reported in this paper A preliminary report has been published elsewhere (7)

EXPERIMENTAL

In order to show that the methylene carbon atom of glycine is incorporated in hemin, two groups of animals were studied, one group consisting of four normal adult rats, and one group consisting of three adult rats which had been rendered anemic by previous phenylhydrazine feeding and which were responding to this anemia by increased hemoglobin production, as evidenced by an increased reticulocyte count

These rats were fed methylene-labeled glycine ($C^{14}H_2NH_2COOH$)[1] of a specific activity of 1 83 microcuries per mg in a single dose in aqueous solution by stomach tube The total dose administered in this way was 2 microcuries, *i e* approximately 1 microcurie per 100 gm of body weight

* This work was performed under contract No W-7401 eng-49 for the Atomic Energy Project at The University of Rochester

[1] This preparation of glycine was synthesized by Dr R Ostwald and kindly made available to us through the courtesy of Dr B M Tolbert of the Radiation Laboratory of the University of California

At varying intervals after the glycine feeding, these animals were anesthetized with sodium pentobarbital and bled as completely as possible from the carotid artery by means of the technique of Buchwald and Hudson (8) This technique was modified first by the injection of a solution of sodium heparin into the femoral vein prior to bleeding from the carotid artery, and second by perfusion with physiological saline solution through the inferior vena cava, this perfusion being continued until the animal died These modifications were adopted in order to secure maximum yields of hemoglobin and in order to free the organs of contained blood

Hemoglobin was isolated by crystallization according to the method of Warburg and Reid (9) Crystalline hemin was prepared as described by Nencki and Zaleski (10) Globin was isolated according to Anson and Mirsky (11) Such globin preparations were carefully washed with acetone and ether and were found by spectroscopic analysis to contain no bound hemin

Method of Analysis—A method developed by Bale and Masters[2] was employed for the determination of C^{14} activity This method involved the conversion of organic material to carbon dioxide by the wet digestion method of Van Slyke and Folch (12) The CO_2 thus produced was then introduced into a 1200 ml ionization chamber, together with inert CO_2, to produce a total pressure of 1 atmosphere The ionization current was read through the use of a ballistic vane electrometer, a version of the dynamic condenser electrometer developed at The University of Rochester The background ionization current is equivalent to approximately 200 C^{14} disintegrations per minute Activities of this order are read to ± 10 per cent accuracy and net activities above twice the background to an accuracy of approximately ± 3 per cent

Results

Glycine Feeding of Normal Rats—Data obtained from the feeding of methylene-labeled glycine to normal, untreated rats are presented in Table I, which shows the activities of hemin, globin, and hemoglobin, each of which was analyzed separately for C^{14} activity

A comparison of the C^{14} content of globin with that of hemin is made for the following reasons (1) a comparison of the respective C^{14} activity of hemin and globin would reveal whether the incorporation of C^{14} in hemin was due to the specific precursor activity of the α-carbon atom of glycine, or whether C^{14} incorporation in hemin was due to a non-specific enrichment of the C^{14} concentration of the body available for the synthesis of proteins and other large molecular components of the tissues, (2) data available on the C^{14} content of globin would permit the establishment of hemin-globin

[2] Bale, W F , and Masters, R E , unpublished method

ratios (shown in the last column of Table I) This ratio may be considered an index of the C^{14} partition between hemin and globin and might possibly show measurable variations under pathological conditions, such as anemia, etc

The C^{14} content of the hemoglobin found checked fairly well with the theoretical value calculated from the C^{14} content of hemin and globin determined independently In making this calculation a molecular weight of 68,000 was assumed for hemoglobin (13), and it was also assumed that 1 molecule of globin is capable of combining with 4 molecules of hemin (14) These theoretical values for hemoglobin are included in Tables I and II The exact reason for the discrepancies is not apparent, although the most

TABLE I

C^{14} Activity of Hemin, Globin, and Hemoglobin in Normal Rats after Feeding $C^{14}H_2NH_2COOH$

Time after glycine feeding	C^{14} activity of hemin		C^{14} activity of globin		C^{14} activity of Hb				Activity ratio, hemin globin gm basis
	Units 10^4 disintegrations per min per gm hemin	Per cent dose in hemin*	Units 10^4 disintegrations per min per gm globin	Per cent dose in globin*	Units 10^4 disintegrations per min per gm. Hb		Per cent dose in Hb*		
					Measured	Calculated†	Measured	Calculated	
days									
1	25 2	0 49	2 84	1 42	3 54	3 69	1 83	1 91	8 9
6	31 0	0 66	4 61	2 57	2 93	5 65	1 69	3 23	6 7
8	18 3	0 29	2 20	0 86	2 69	2 82	1 10	1 15	8 3
8	17 4	0 46	2 31	1 56	2 04	2 87	1 43	2 02	7 5

* On the basis of total circulating blood

† On the basis of hemin and globin measurements

likely cause for this disagreement is contamination of hemoglobin or globin samples In both groups of animals, one of the calculated hemoglobin values deviates markedly from the value found, in each case the measured value being lower than the calculated value It is conceivable that a low activity contaminant, *e g* stroma, precipitated with hemoglobin, thus bringing about the observed disagreement between the calculated and measured values

In Table I are also shown calculations of the percentage of the total dose of C^{14} incorporated in hemin, globin, and hemoglobin These percentages were calculated from independent colorimetric hemoglobin determinations[3] before the animals were sacrificed or on the assumption that the hemoglobin of the rat represents 13 8 per cent of the total rat blood, which in turn

[3] Acid hematin was determined with the photoelectric colorimeter of Klett and Summerson

amounts to 7 per cent of the total body weight The value assumed for hemoglobin is based upon that published by Thewlis and Meyer (15) and checks with a small series of rats used in our laboratory[4] In general, 0 5 per cent of the total dose of C^{14} administered was incorporated in hemin

Glycine Feeding of Phenylhydrazine-Treated Rats—Three rats were each given 24 mg of phenylhydrazine hydrochloride by stomach tube on 2 alternate days, resulting in a depression of the erythrocyte count These animals were then allowed to enter the recovery period, in the course of which the reticulocyte count reached a level of 15 to 20 per cent When this level was reached, methylene-labeled glycine was administered

Since these animals were synthesizing hemoglobin at a more rapid rate than normal rats, it is not surprising to find that the hemoglobin contained considerably higher concentrations of C^{14} This is shown in Table II It

TABLE II

C^{14} Activity of Hemin, Globin, and Hemoglobin of Phenylhydrazine-Treated Rats after Feeding $C^{14}H_2NH_2COOH$

Time after glycine feeding	C^{14} activity of hemin units, 10^4 disintegrations per min per gm hemin	C^{14} activity of globin, units, 10^4 disintegrations per min per gm globin	C^{14} activity of Hb, units, 10^4 disintegration per min per gm Hb		Activity ratio, hemin globin, gm basis
			Measured	Calculated*	
days					
1	52 2	7 18	3 42	8 9	7 3
4	58 5	8 24	10 9	10 2	7 1
5	160 0	16 6	23 5	22 1	9 6

* Calculated on the basis of hemin and globin measurements

is interesting to note that the ratio of hemin to globin, expressed on a gm to gm basis, remains fairly constant and agrees quite well with that of the normal animals This suggests that the partition of C^{14} in normal animals and in animals recovering from phenylhydrazine anemia varied within similar, narrow limits and was of the same order of magnitude It cannot be said with certainty at this time whether the ratio of newly formed hemin to newly formed globin is constant under all conditions There are indications, however, that under certain nutritional conditions[5] this hemin to globin ratio is altered extensively

The significance of the hemin-globin ratios cannot be completely assessed at this time However, the hemin-globin ratio is considered to reflect the rates at which hemin and globin are synthesized and the differences between

[4] For the blood volume, the assumed value is based on that cited by Griffith and Farris (16)

[5] Unpublished experiments of the authors

these respective rates The rates of incorporation of glycine in hemin and globin could be assessed by comparing the C^{14} activity per glycine residue incorporated in hemin and in globin Such a comparison reveals that the C^{14} activity per glycine residue is approximately $1\frac{1}{2}$ times as high for hemin as for globin, suggesting that the rate of hemin synthesis is slightly faster than the rate of globin synthesis

For the purpose of making an approximate preliminary calculation, it is assumed that 75 per cent of the total C^{14} activity of globin is present as glycine residues,[6] and that there are 50 glycine residues per mole of globin [7] It is furthermore assumed that 4 glycine residues are incorporated in every molecule of newly formed hemin

DISCUSSION

The experiments reported in this paper indicate that the methylene carbon atom of glycine is incorporated into the hemin molecule, and that glycine labeled with C^{14} in its α-carbon atom acts as a precursor of the tetrapyrrole structure of hemin Because of the finding that the amount of C^{14} activity of hemin is significantly greater than that of globin, it may be concluded that the incorporation of glycine into hemin is a process which does not depend upon the enrichment of the C^{14} concentration of the body Thus, it appears probable that the α-carbon atom of glycine is incorporated directly into the hemin molecule The mechanism of this reaction is at present unknown

Shemin and Rittenberg (4) have pointed out that glycine may participate in hemin synthesis in a manner analogous to a reaction recently described by Fischer and Fink (18) in which a pyrrole-like substance is formed as a result of the condensation of glycine and a β-ketoaldehyde Such a concept is tenable even in view of the report that the carboxyl carbon atom of glycine is not incorporated in hemin (19) [8] If the Fischer and Fink reaction is operative in hemin synthesis, it must be assumed that the carboxyl group is removed at some point during the condensation reaction or after the formation of the pyrrole ring

It appears more likely that the carboxyl group is removed after the pyrrole ring has been formed, since no enzyme system capable of decarboxylating glycine has so far been discovered, the possible exception being the fermentation of glycine by *Diplococcus glycinophilus* (20, 21) This point

[6] It is quite conceivable that the entire C^{14} activity of globin does not reside in the glycine residues, but that the C^{14} activity of other amino acids also contributes to the C^{14} activity of globin

[7] The glycine content of rat hemoglobin is assumed to be analogous to that of horse hemoglobin as determined by Shemin and Foster (17)

[8] Radin, N , Rittenberg, D , and Shemin, D , personal communication

of view is also supported by the experiment of Lorber and Olsen (22) who reported that no appreciable decarboxylation of glycine, labeled with C^{13} in the carboxyl group, takes place in isolated mammalian heart preparations Furthermore, the hemin of rats to which $CaC^{14}O_3$ has been administered contains significant amounts of C^{14} (23) Thus, one would expect that, if carboxyl-labeled glycine were decarboxylated, the CO_2 thus formed could contribute to hemin synthesis, provided that its concentration was high enough

Indirect evidence that the carbon-nitrogen bond in glycine remains intact is derived from the observation of Shemin and Rittenberg (3) that N^{15}-containing ammonium citrate fed to rats is not incorporated into hemin These experiments also suggest that the oxidative deamination described by Ratner, Nocito, and Green (24) for a variety of tissues does not play a significant rôle as far as glycine utilization for hemin synthesis is concerned

Suitable methods for the degradation of the hemin molecule are as yet not available For this reason it has not been possible to determine the distribution of C^{14} in the tetrapyrrole structure of hemin Attempts to develop such methods are now in progress in this laboratory

SUMMARY

1 It has been shown that α-carbon-labeled glycine is incorporated into hemin and globin of normal and phenylhydrazine-treated animals, and that the hemoglobin of phenylhydrazine-treated animals contains higher concentrations of C^{14} activity than that of normal animals

2 The data presented suggest that the partition of C^{14} activity between hemin and globin is essentially the same in normal animals and in animals recovering from an anemia produced by phenylhydrazine feeding

The authors wish to express their thanks to Dr William F Bale for his continuing interest in this investigation

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THE EFFECT OF TESTOSTERONE AND METHYLTESTOSTERONE ON GUANIDOACETIC ACID, CREATINE, AND CREATININE IN PLASMA AND URINE*

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Since the major route of creatine formation in the animal body appears to involve the intermediate formation of guanidoacetic acid, methylated steroids such as methyltestosterone could cause the repeatedly observed (1, 2) increase in production of creatine by one of three possible mechanisms (*a*) by transferring methyl groups to guanidoacetic acid, (*b*) by increasing production of guanidoacetic acid, or (*c*) by catalyzing some other system of creatine synthesis

Wilkins and Fleishmann (3) in 1945 were able to demonstrate that only the methylated derivatives of the C_{19} steroid nucleus were effective in producing creatinurea Methyltestosterone, methylandrostanediol-3,17, and methylandrostene-5-diol-3,17 all caused creatinurea, while ethyltestosterone and a number of other non-methylated steroids were found to be inactive As a result of this work the idea was proposed that methylated steroids may act as catalysts for the process of methylating guanidoacetic acid to creatine

The work of Borsook and Dubnoff (4, 5), du Vigneaud *et al* (6), and Block and Schoenheimer (7) has established the probable mechanism of creatine formation as follows (*a*) guanidoacetic acid is formed in the kidney from glycine and arginine and (*b*) the guanidoacetic acid is methylated by methionine, principally in the liver Borsook *et al* (8) demonstrated that administration of arginine and glycine to man led to a marked increase in guanidoacetic acid in the urine Simmonds and du Vigneaud (9) furnished indisputable proof that the methyl group of methionine gives rise to creatinine in man It therefore appears that this proposed mechanism applies to man

If the rôle of catalyst proposed by Wilkins and Fleishmann were correct, it would follow that the increase in creatine levels should bear certain relations to the levels of guanidoacetic acid Two alternatives appear possible In the first case methylation of guanidoacetic acid could be accelerated

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without an increase in its production, if this were the mechanism, the guanidoacetic acid levels of the blood and urine should decrease in direct relation to the increase in the creatine levels In the second instance, the increased removal of guanidoacetic acid from the system by methylation could lead to increased guanidoacetic acid synthesis, thus its level would remain approximately constant while the creatine levels increased

The present work was initiated to determine the plausibility of the proposed catalyst mechanism Accordingly, normal male and female subjects were given methyltestosterone orally and the guanidoacetic acid and creatine levels of both the blood and urine were determined over an extended time If the proposed mechanism operated, the levels of the precursor should remain constant or decrease while creatine increased Similarly, the creatine and guanidoacetic acid levels of the blood and urine have been determined for normal male subjects given testosterone by injection, by implantation, and by oral administration This series has served to check the possibility that the steroid nucleus as such would influence the guanidoacetic acid levels

Methods

Dogs, rabbits, guinea pigs, rats, pigs, and monkeys have failed to show creatinurea following methyltestosterone administration Consequently, it was necessary to use humans for this study The selected subjects were placed on low creatine diets 48 hours later the first of three consecutive 24 hour urine samples was collected Blood samples were drawn at the end of each 24 hour collection period Following collections of urine and blood, patients were permitted to return to normal diets These normal diets were maintained until 48 hours before the next collections when the low creatine diet was again employed

Administration of methyltestosterone or testosterone was begun on the last day of the first collection period In the case of methyltestosterone, one 10 mg tablet was given before each meal and two before retiring, making a total of 50 mg per day In the series utilizing testosterone propionate one subject was given 50 mg of testosterone propionate in peanut oil orally, two subjects were given intramuscular injections of 25 mg of the same compound every 2 days, and one subject had four 150 mg pellets of testosterone propionate implanted intramuscularly in the left infrascapular region

The plasma and urine were analyzed for preformed and total creatinine (10) The method of Peters was altered by lengthening the autoclave period to 45 minutes to complete the conversion of creatine to creatinine at this altitude, and by diluting the urine specimens to constant volume rather than to constant specific gravity This latter change was made only for convenience in the analysis, since variation in specific gravity was not large

Guanidoacetic acid was estimated by the method of Dubnoff and Borsook (11) This method was modified slightly in that the samples were warmed to room temperature by rubbing the sample container between the hands for 1 minute before colorimetric readings were made The creatinine and guanidoacetic acid determinations were run in duplicate on each sample

TABLE I

Guanidoacetic Acid Recoveries with Permutit Separation (Average of Duplicate Determinations)

Test No	Arginine added	Guanidoacetic acid added	Guanidoacetic acid recovered
	mg per 100 cc	*mg per 100 cc*	*mg per 100 cc*
1	0	0 125	0 121
2	0	0 250	0 251
3	0	0 50	0 50
4	0	1 00	1 02
5	30	3 0	2 72
6	30	1 0	0 98
7	20	1 0	1 0
8	10	3 0	2 70
9	10	1 0	1 04
10	0	0	0

TABLE II

Creatinine Recoveries after Autoclaving with Guanidoacetic Acid (Average of Duplicate Determinations)

Test No	Creatinine added	Guanidoacetic acid added	Creatinine recovered
	mg per 100 cc	*mg per 100 cc*	*mg per 100 cc*
1	0 83	0	0 84
2	0 83	0 25	0 83
3	0 83	0 50	0 84
4	0 83	1 00	0 84

Results

Preliminary Measurements—Since some workers (12, 13) have experienced difficulty with the method for the determination of guanidoacetic acid as proposed by Dubnoff and Borsook, recovery measurements were made utilizing varying quantities of guanidoacetic acid and arginine The results are given in Table I These data show that satisfactory separation of the arginine and guanidoacetic acid was obtained with the permutit samples used Further, added guanidoacetic acid could be recovered with a precision of approximately 5 per cent or better in the range of physiological concentrations

If present in sufficient quantity, the anhydride of guanidoacetic acid, formed during autoclaving, could be an interfering substance in the creatine

TABLE III

Creatine, Creatinine and Guanidoacetic Acid Levels in Plasma and Urine of Male and Female Subjects Given 50 mg. of Methyltestosterone Daily

Each value represents duplicate samples on 3 consecutive days

	Days on experiment	Male			Females		
		M J B	L B	B H L	L A	M H	M S
Plasma creatine, *mg per 100 cc*	0				0 33	0 27	0 52
	13	0 45	0 35	0 37	0 44	0 40	0 61
	22*				0 69	0 83	1 05
	28	0 50	0 48	0 59			
	42†	0 80	0 61	0 74			
	56	0 49	0 51	0 33			
	72	0 57	0 42	0 42			
Urine creatine, *gm per day*	0				0 046	0 032	0 057
	13	0 046	0 053	0 047	0 036	0 080	0 065
	22*				0 255	0 402	0 523
	28	0 047	0 048	0 053			
	42†	0 183	0 160	0 134			
	56	0 029	0 055	0 038			
	72	0 040	0 040	0 033			
Plasma guanidoacetic acid, *mg per 100 cc*	0	0 26	0 28	0 24	0 34	0 26	0 25
	13	0 24	0 27	0 26	0 46	0 34	0 54
	22*				0 45	0 43	0 45
	28	0 26	0 22	0 19			
	42†	0 42	0 40	0 38			
	56	0 39	0 25	0 27			
Urine guanidoacetic acid, *gm per day*	0	0 036	0 024	0 016	0 026	0 037	0 028
	13	0 073	0 041	0 046	0 051	0 105	0 067
	22*				0 118	0 272	0 175
	28	0 065	0 051	0 070			
	42†	0 114	0 085	0 114			
	56	0 059	0 051	0 039			
	72	0 061	0 040	0 034			
Plasma creatinine, *mg per 100 cc*	0				0 88	0 92	0 84
	13	1 37	1 34	1 36	1 02	1 03	1 05
	22*				1 00	1 04	1 05
	28	1 17	1 31	1 21			
	42†	1 19	1 41	1 40			
	56	1 26	1 42	1 47			
	72	1 41	1 50	1 50			

TABLE III—*Concluded*

	Days on experiment	Males			Females		
		M J B	L B	B H L	L A	M H	M S
Urine creatinine, *gm per day*	0				0 907	1 182	1 143
	13	1 776	1 873	1 812	1 150	1 368	1 273
	22*				0 955	1 307	1 302
	28	1 763	1 934	1 967			
	42†	1 767	2 094	2 112			
	56	2 037	2 067	2 147			
	72	1 927	2 167	2 240			

* Methyltestosterone withdrawn from female subjects
† Methyltestosterone withdrawn from male subjects

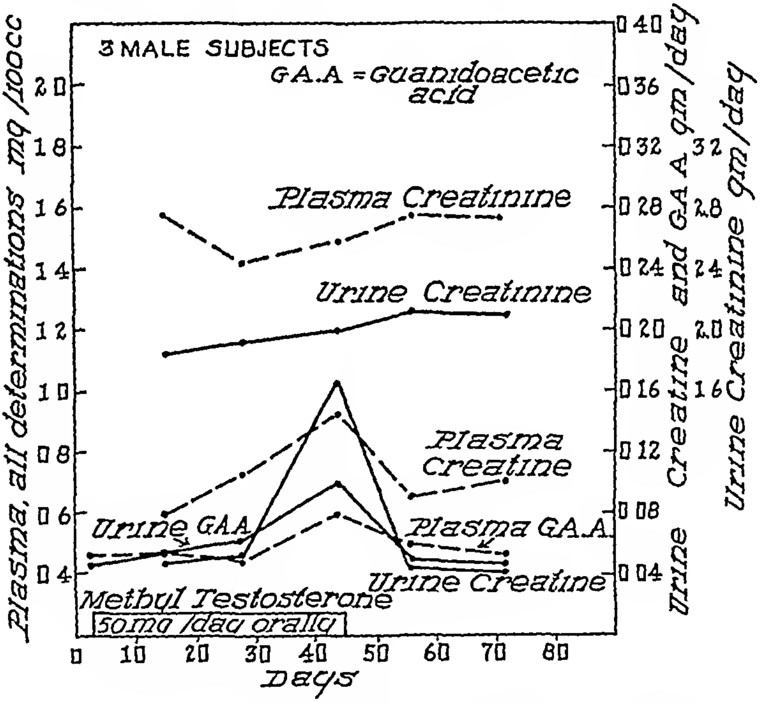

FIG 1 Average guanidoacetic acid, creatine, and creatinine levels of plasma and urine for three male subjects given 50 mg per day of methyltestosterone orally

determination To check this possibility creatinine determinations were made after 45 minutes autoclaving on samples containing varying amounts of guanidoacetic acid and creatinine The results of these experiments are given in Table II These data show that guanidoacetic acid in the quanti-

ties encountered in this work does not represent an interfering substance in the creatine determination The N-methyl group of creatinine is known to prevent the interference of this compound in the determination of guanidoacetic acid (14)

Studies Involving Methyltestosterone—The plasma and urine levels of creatine and guanidoacetic acid were determined in three normal men and three normal women receiving methyltestosterone The results are given in Table III, and the average results are shown in Figs 1 and 2 In the

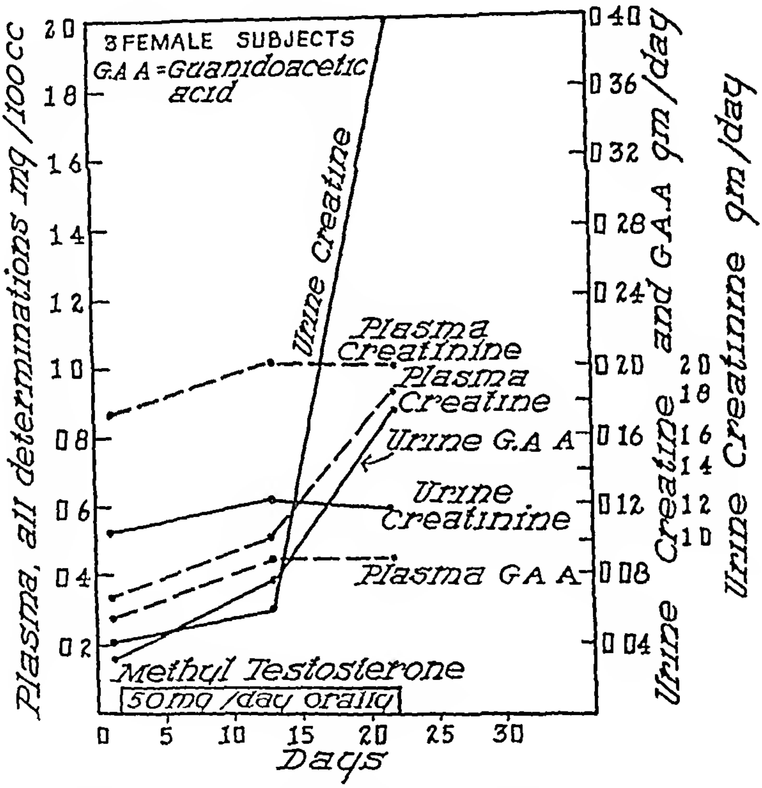

FIG 2 Average guanidoacetic acid, creatine, and creatinine levels of plasma and urine for three female subjects given 50 mg per day of methyltestosterone orally

cases involving the men 50 mg of methyltestosterone were administered daily for 43 days Significant increases in urine levels of creatine and guanidoacetic acid occurred after an initial delay of approximately 20 days The plasma levels were significantly higher at 20 days than at 10 days Original plasma levels were not determined in this series 10 days following cessation of methyltestosterone administration, all levels had returned to normal

The female subjects received 50 mg of methyltestosterone a day for

20 days. There was no significant delay before the elevation of the creatine and guanidoacetic acid levels of plasma. The urine guanidoacetic acid level also rose significantly, until the plasma level passed the renal threshold of 0.6 to 0.7 mg. per 100 cc. The values of all constituents except plasma

TABLE IV

Creatine, Creatinine, and Guanidoacetic Acid Levels in Male Subjects Given Testosterone Propionate

Each value represents duplicate samples on 3 consecutive days

	Days on experiment	J. W.	I. K.	B. H. L.	M. J. B.
Plasma creatine, *mg. per 100 cc.*	0	0.37	0.34	0.29	0.37
	13	0.34	0.32	0.30	0.42
	22	0.28	0.26	0.28	0.34
	41	0.31	0.33	0.26	0.38
Urine creatine, *gm. per day*	0	0.033	0.025	0.027	0.030
	13	0.040	0.026	0.030	0.023
	22	0.035	0.015	0.005	0.010
	41	0	0	0	0
Plasma guanidoacetic acid, *mg. per 100 cc.*	0	0.44	0.34	0.30	0.27
	13	0.45	0.34	0.27	0.27
	22	0.55	0.32	0.30	0.31
	41	0.55	0.36	0.35	0.34
Urine guanidoacetic acid, *gm. per day*	0	0.048	0.052	0.036	0.057
	13	0.045	0.047	0.028	0.058
	22	0.051	0.042	0.033	0.058
	41	0.050	0.057	0.031	0.062
Plasma creatinine, *mg. per 100 cc.*	0	1.49	1.21	1.33	1.26
	13	1.52	1.33	1.62	1.57
	22	1.46	1.23	1.28	1.26
	41	1.50	1.27	1.27	1.35
Urine creatinine, *gm. per day*	0	1.470	1.180	1.847	1.350
	13	1.470	1.512	1.845	1.895
	22	1.567	1.678	2.030	1.981
	41	1.690	1.600	1.710	1.800

guanidoacetic acid rose throughout the 20 day period of treatment. The leveling off of the plasma guanidoacetic acid at approximately 0.45 mg. per 100 cc. is thought to represent the maximal renal reabsorption level of this compound.

Studies Involving Testosterone Propionate—While the action of testoster-

one propionate on the creatine levels of normal subjects is well known, no information has been available on the guanidoacetic acid levels. Consequently, the plasma and urine levels of creatine and guanidoacetic acid were determined for four normal male patients receiving testosterone propionate as described. By using intramuscular injection, implantation, and oral administration of testosterone propionate, it was possible to eliminate the variable of route of administration from the measurements. The

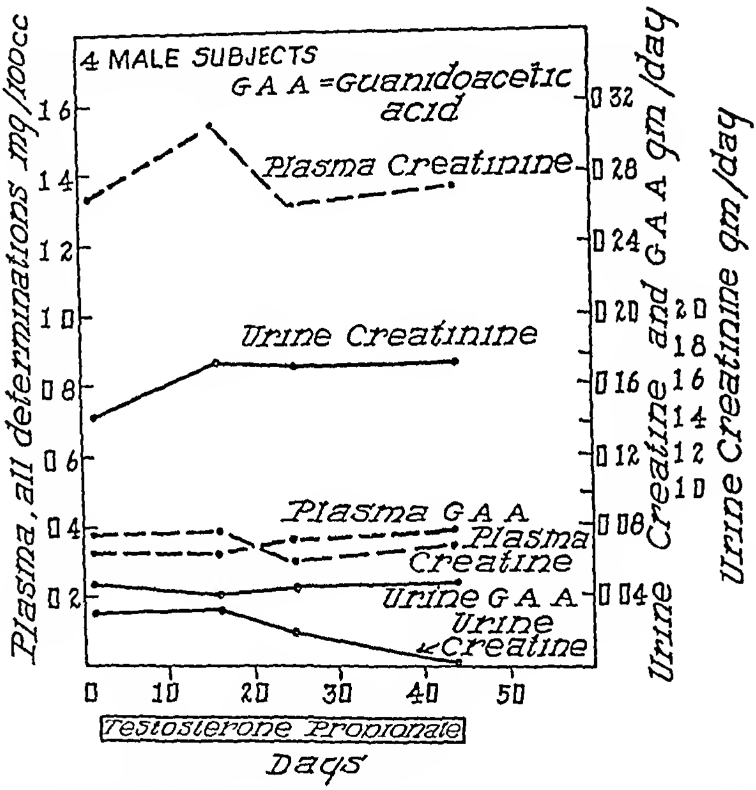

FIG 3 Average guanidoacetic acid, creatine, and creatinine levels of plasma and urine for four male subjects given testosterone propionate by various routes of administration. The route of administration did not affect results

results of these experiments are given in Table IV and the average results shown in Fig 3

These data show that there was no significant change in the plasma creatine and guanidoacetic acid levels and the urine guanidoacetic acid level during the course of the experiment. After an initial 10 day delay the urine creatine level decreased in all subjects regardless of the route of administration of the testosterone propionate. All other levels remained constant throughout the administration period

DISCUSSION

The results of this study lead to the elimination of the previously proposed rôle of methyltestosterone in creatine metabolism Since the guanidoacetic acid levels as well as the creatine levels are elevated following methyltestosterone administration, the rôle of a methyl-transferring agent previously suggested for methyltestosterone is no longer tenable The elevation of the guanidoacetic acid levels of plasma and urine indicate that the methyltestosterone must act primarily at some stage prior to guanidoacetic acid methylation The steroid nucleus as such was obviously not involved in the process of increased formation of guanidoacetic acid, since testosterone was ineffective

The evidence of Samuels, Sellers, and McCaulay (15) that acute renal damage decreases or prevents the effect of methyltestosterone indicates that the kidney is the site of the increased formation of the guanidoacetic acid, apparently the primary effect of methyltestosterone in creatine metabolism This is additional evidence that the order of synthesis demonstrated in rats is also that followed in the human Additional statements of the action of methyltestosterone must await the elucidation of the particular system catalyzed by the methylated steroids

SUMMARY

1 Guanidoacetic acid and creatine levels have been determined in normal male and female subjects who had received methyltestosterone The guanidoacetic acid and creatine levels were found to increase in the same general fashion in both plasma and urine

2 The possibility of the steroid nucleus *per se* bringing about increased guanidoacetic acid production has been eliminated

3 Methyltestosterone affects some process associated with the synthesis of guanidoacetic acid, rather than its methylation alone

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CHROMATOGRAPHY OF AMINO ACIDS ON STARCH COLUMNS SEPARATION OF PHENYLALANINE, LEUCINE, ISOLEUCINE, METHIONINE, TYROSINE, AND VALINE

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In the past few years, several methods for the chromatographic fractionation of mixtures of amino acids have been introduced by Martin and Synge and their coworkers Chromatography of the N-acetyl amino acids on silica gel columns has been employed by Martin and Synge (1, 2), and has been further studied by Tristram (3) The separation of the free amino acids by chromatography on paper, following its introduction by Consden, Gordon, and Martin (4), has found wide use A third method, chromatography on starch columns, has been the subject of a note by Elsden and Synge (5), and was used by Synge (6) in qualitative experiments with partial hydrolysates of gramicidin

It appeared that the starch column should possess characteristics rendering it the technique of choice for some types of problems As Synge (6) pointed out, free amino acids and peptides may be chromatographed on starch, blocking of the amino group by acetylation being unnecessary In addition, it was to be anticipated that mixtures should be fractionable in sufficient quantity with starch columns to permit subsequent examination of the components by conventional microchemical techniques The procedure also appeared to be one which could be developed into a quantitative method for amino acid analysis

The investigations described in this communication deal with developmental work on the chromatography of amino acids on starch columns and with specific methods for the separation and quantitative determination of phenylalanine, leucine, isoleucine, methionine, valine, and tyrosine in protein hydrolysates Studies on the remaining common amino acids form the subject of a paper now in preparation

In the fractionation of partial hydrolysates of gramicidin on starch columns, Synge (6) collected the effluent from the column in relatively large fractions and spot-tested each qualitatively with ninhydrin-impregnated paper The volume of a fraction was adjusted to include substances within a given range of zone rates Each fraction was worked up individually and its contents examined

In the present investigations, the effluent has been collected in a regular

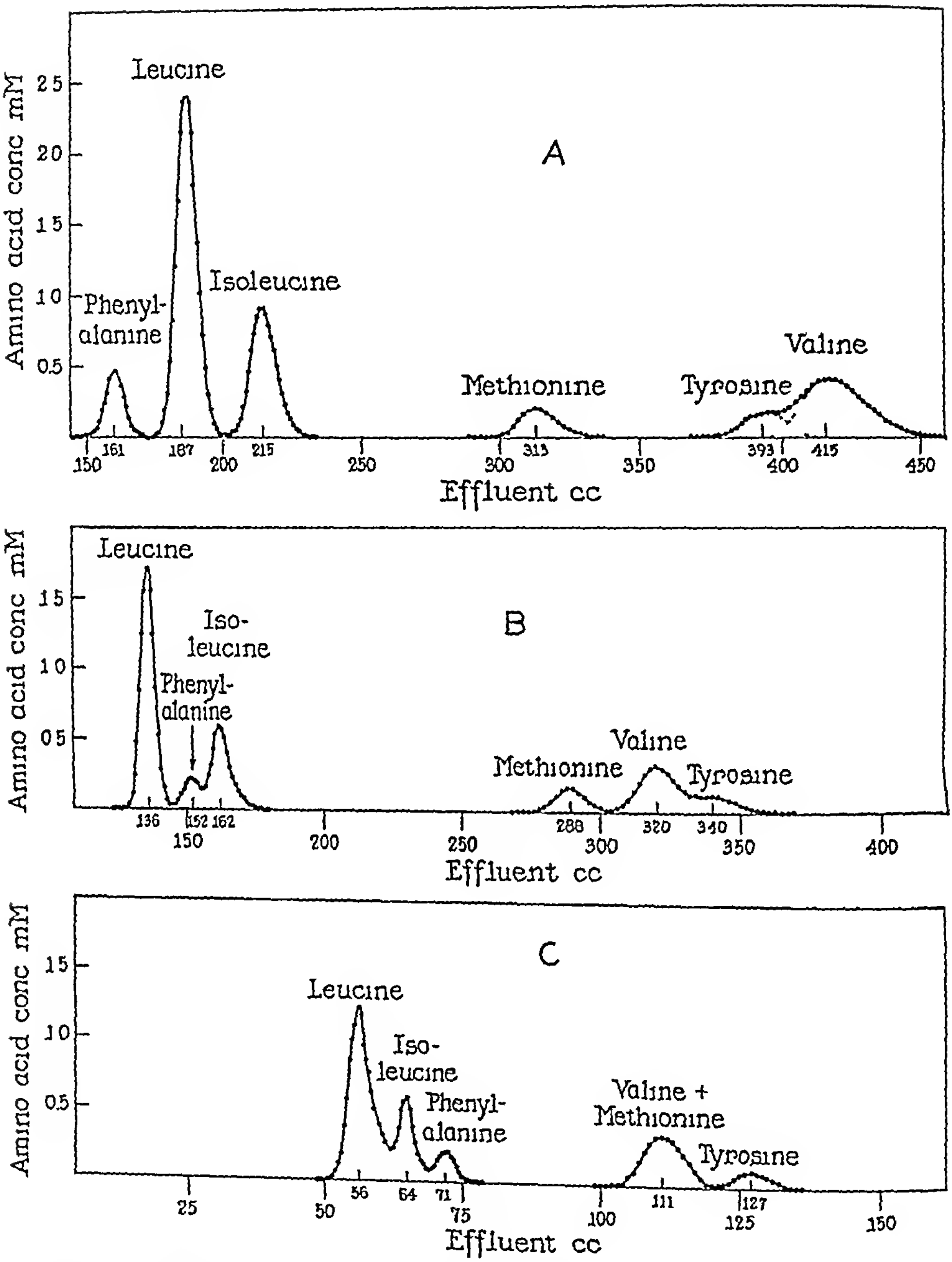

FIG 1 Separation of amino acids from a synthetic mixture containing eighteen amino acids and NH_4Cl, corresponding in composition to an acid hydrolysate of β-lactoglobulin Amino acid concentrations are given in leucine equivalents (11) *A*, solvent, 1 1 0 288 *n*-butanol-benzyl alcohol-water Column, 52 gm of starch (anhydrous), diameter, 1 9 cm , height, 30 cm Sample, 20 mg of synthetic mixture *B*, solvent, *n* butanol-15 per cent water Column, 55 gm of starch (anhydrous), diameter, 1 9 cm , height, 29 cm Sample, 10 mg of synthetic mixture *C*, solvent, *n*-butanol-17 per cent 0 57 N HCl Column, 55 gm of starch (anhydrous), diameter, 1 9 cm , height, 28 cm Sample, 5 mg of synthetic mixture

series of small fractions of known volume, and the fractions have been analyzed quantitatively rather than qualitatively The data thus obtained permit the construction of effluent concentration curves which reveal the detailed behavior and the full resolving power of the column The effluent fractions have been collected with the aid of a specially constructed, fully automatic fraction-collecting machine, and the fractions have been analyzed quantitatively for amino acids by means of a photometric ninhydrin method developed for this purpose

Effluent concentration curves showing the behavior of six amino acids in three solvents are given in Fig 1 These amino acids are the first to emerge from a starch column when the sample fractionated consists of a known mixture of eighteen amino acids and ammonium chloride made up to simulate the composition of an acid hydrolysate of β-lactoglobulin (*cf* data of Brand *et al* (7)) The procedure employed to obtain curves of the type shown in Fig 1 is given in the experimental section, followed by a discussion of the influence of variables on the process and by a summary of the results obtained with protein hydrolysates

Procedure

Preparation of Starch Column—The chromatograph tubes used in these experiments were of the Zechmeister-Cholnoky type with ground joints and sintered glass plates[1] The following directions are for the preparation of a 30 cm starch column in a tube of 0 9 cm inner diameter and 40 cm in length Direct proportionality factors can be used for columns of other dimensions The columns were prepared and run in an air-conditioned room at 25° ± 0 5° There have been no indications that a constant temperature room is essential Recent experiments have indicated that satisfactory results can be obtained at room temperatures of 25° ± 5°

The starch employed in this investigation was potato starch powder manufactured by Morningstar Nicol, Inc[2] Different batches procured from the same manufacturer have given fully reproducible results In order to obtain the correct tightness of column packing, the water content of the starch at the time the column is prepared must be carefully controlled The moisture content of the air-dried product is determined by drying a sample to constant weight at 110° at atmospheric pressure The moisture content of starch may vary with the atmospheric humidity, and should be checked periodically An air-dried sample corresponding to 13 4 gm of

[1] Purchased from the Scientific Glass Apparatus Company, Bloomfield, New Jersey, catalogue No J-1664 A length of 40 cm above the sintered plate must be specified for use with starch columns 30 cm in height

[2] Manufactured from white potatoes by Morningstar Nicol, Inc , New York, and purchased from Amend Drug and Chemical Company, Inc , New York

anhydrous starch is weighed out For the starch used in these experiments, the sample was suspended in 25 cc of dry butanol in which enough water had been dissolved to bring the total water present to 30 per cent of the dry weight of the starch For starch containing 20 per cent moisture, 16 8 gm were weighed out and suspended in butanol containing 0 7 cc of water The starch is stirred thoroughly with a glass rod until a uniform suspension free from lumps is obtained During this time the starch adsorbs most of the water present in the butanol The suspension is poured into the upright chromatograph tube through a funnel possessing a tip bent to touch the side of the tube In this manner, the suspension flows down the side of the tube without incorporation of air bubbles For chromatograph tubes up to 2 cm in diameter, a 20 cm extension of glass tubing of the same diameter is attached to the top of the tube during the pouring process This extension is necessary to accommodate the full volume of the slurry The connection is secured glass to glass with rubber tubing For tubes possessing a diameter larger than 2 cm, the columns can be packed in portions without an extension by first pouring two-thirds of the slurry and adding the remainder after the first portion has settled to constant height

After the suspension has been poured into the tube, the column is placed under an air pressure of 5 to 7 cm of mercury[3] The starch settles slowly over a period of 1 to 3 hours If the moisture content of the starch has been correctly adjusted, a sharp settling line can be seen to move steadily up the tube when the column is illuminated from behind with a strong light When the starch has packed to a constant height, the extension tube, if one was used, is removed and the butanol remaining on the surface of the column is withdrawn with a pipette attached to a rubber bulb The solvent with which the chromatogram is to be run is added carefully to the top of the column without disturbing the surface of the starch The tube is filled to within 5 cm of the top A 125 cc separatory funnel filled with solvent is attached to the column by a micro rubber stopper (Arthur H Thomas Company, catalogue No 8823-A) through which the constricted tip extends about 2 cm An air lock is thus formed between the solvent and the stopper in order to avoid contact of the liquid with the rubber A pressure of 8 cm of mercury is applied to the top of the separatory funnel and main-

[3] For routine use, reducing valves are installed on the compressed air lines Air filtered through a ½ inch Logan airdifier (Crane and Company, 47-30 29th Street Long Island City, New York) is drawn at 70 pounds pressure through a reducing valve type R-79 (Linde Air Products Company, 205 East 42nd Street, New York) The outlet gage on the valve is replaced by a test gage reading from 0 to 25 cm of mercury, 2½ inches in diameter, procured from the Factory Products Company, 16: Meserole Avenue, Brooklyn 22 A T-tube with a rubber tube and screw clamp is placed in the line to the chromatograph tube to provide a constant "bleeder"

tained until about 50 cc of solvent have passed through the column This procedure, which usually requires about 36 hours, permits the starch to adsorb enough water to become equilibrated with the wet organic solvent The resulting swelling of the starch may cause the surface of the column to rise 1 to 2 cm If butanol-benzyl alcohol solvent mixtures have been employed, the column will gradually become translucent during the equilibration process

After the starch columns have become fully equilibrated, they are operated under a pressure of 15 cm of mercury Pressure in excess of 8 cm during the equilibration period, or of 15 cm during operation, may cause the generation of air bubbles in the lower half of the column, and, therefore, should not be used The presence of a small number of air bubbles within a few cm of the sintered plate is frequently noted, and does not interfere with the results

The degree of tightness of the column packing must be checked by measurement of the flow rate after equilibration with butanol-water or butanol-benzyl alcohol-water A satisfactory column, 0 9 cm in diameter and 30 cm in height, should possess a flow rate of 1 25 to 1 50 cc per hour at 15 cm of mercury pressure (2 0 to 2 4 cc per hour per sq cm of cross-sectional area on a column 30 cm in height) Preparations of potato starch from different sources may vary in average particle size, which can affect the amount of water which should be present when the column is poured In general, it can be suggested that if too fast or too slow a flow is obtained, the water content at the time of pouring should be diminished or increased to establish the optimum conditions for the preparation being used

The surface of the column must be firmly packed before the sample is added The solvent is removed to within 1 to 2 mm of the starch The remaining liquid is driven down at 7 cm pressure until it clears the surface The pressure is maintained for several minutes until the starch has fallen 1 to 2 mm If any scum from the solvents has collected on the surface of the column, the top 1 mm of starch should be removed with a silver spatula The surface is tamped and leveled with a flat tipped 6 mm glass rod Air pressure is reapplied until the surface is fairly dry as evidenced by a frosty appearance The surface is tamped again with the glass rod, and the process repeated until a firm, smooth surface which does not cling to the rod is obtained For columns larger than 0 9 cm in diameter, the preliminary packing of the surface can be performed with a spatula and the final smoothing of the surface with a glass or stainless steel plunger possessing a diameter a few mm less than that of the chromatograph tube Care must be taken while preparing the surface not to continue the air pressure for too long a period If this is done, the starch will pull away from the

walls of the tube and air bubbles will be seen below the surface. If the bubbles extend only a few mm., the column still gives satisfactory results. If the bubbles extend down several cm., the column should be discarded.

Except when strongly acidic solvents are being used, starch columns require treatment with 8-hydroxyquinoline to remove traces of interfering metal ions. For columns 0.9 cm. in diameter, a solution of 25 mg. of 8-hydroxyquinoline (Merck) in 2.5 cc. of the solvent used on the column is added to the tube and driven into the starch at 15 cm. pressure. When this solution has just cleared the surface, fresh solvent is added and run in until the yellowish green band of hydroxyquinoline is at least 5 cm. below the surface.

The column is then ready for the addition of the sample. If not used immediately, the columns can be allowed to drip under gravity or can be kept for at least several weeks with the solvent and separatory funnel (stop-cock closed) on the top and with the tip immersed in a test-tube filled with solvent. At the end of the experiment, the starch is conveniently removed from the narrow chromatograph tubes by a jet of water from stiff tubing (Tygon). Columns run with the solvents described in this communication should not be used more than once. The exception to this statement is the case in which the mixture applied to the column contains only the amino acids shown in Fig. 1 (or tryptophan) and is free of acids or neutral salts.

Addition of Amino Acid Sample to Column—For the curves shown in Fig. 1, an amino acid mixture[4] simulating the composition of an acid hydrolysate of β-lactoglobulin (7) was employed. A total of about 1 gm. of amino acids was dissolved in 1.5 cc. of approximately 6 N HCl and made up to a volume of 10 cc. with water. Methionine and cysteine were not included in this stock mixture, although subsequent experiments have shown that methionine may be incorporated without risk of deterioration on storage of the solution at 3°. Immediately before an experiment, the appropriate quantities of methionine and cysteine hydrochloride were made to a volume of 10 cc. with 0.1 N HCl. To a 10 cc. volumetric flask, 0.5 cc. samples of each of the aqueous solutions referred to above were added and the mixture was

[4] All the amino acids employed were checked for correct elementary analysis (carbon, hydrogen, and nitrogen). Specific rotations were measured on the L-amino acids. The following preparations were used: DL-leucine, DL-isoleucine, DL-methionine, DL-valine, DL-aspartic acid, DL-threonine, glycine, L-arginine hydrochloride, L-cysteine hydrochloride, and L-cystine (all Merck), L-tyrosine and L-glutamic acid (Corn Products, recrystallized), L-proline from gelatin (8), L-alanine, L-serine, L-leucine, L-phenylalanine, L-histidine·HCl·H_2O, and L-lysine dihydrochloride prepared from protein hydrolysates (9). We are indebted to Dr. Erwin Brand for samples of L-tryptophan and L-valine, to Dr. Karl Folkers of Merck and Company, Inc., for a sample of L-methionine, and to Dr. E. E. Howe of the same company for the DL-leucine and DL-isoleucine.

made to volume with *dry* butanol or 1 1 butanol-benzyl alcohol If difficulty is encountered in dissolving the amino acid mixture upon shaking, it may be necessary to add 0 2 to 0 4 cc of ethanol and 1 or 2 drops of 6 N HCl before the solution is diluted to volume For a column 0 9 cm in diameter 0 5 cc[5] of the resulting solution, corresponding to about 2 5 mg of total amino acids, is added to the top of the column, care being taken not to disturb the surface of the starch The experiments illustrated in Fig 1 were run on a column 1 9 cm in diameter with a 2 cc sample of the amino acid solution

Air pressure (15 cm) is applied and the sample driven into the column After the liquid has reached the level of the starch, 0 2 cc of solvent is added to rinse down the walls of the tube The wash solution is forced into the column under pressure The washing operation is carried out three times Solvent is added over the column, the reservoir connected, and air pressure of 15 cm is applied Measurement of the effluent volumes recorded in Figs 1, 3, and 4 was started at this time A graduate is placed under the column and an appropriate fore fraction is usually taken before the column is placed on the fraction-collecting machine For the first run on an unknown sample, a fore fraction is not taken and the curve is checked from the beginning for possible peaks ahead of phenylalanine and leucine

The developmental work to establish the positions of the peaks with different solvents was done with synthetic mixtures containing one to six components A mixture containing 50 to 150 mg of each component was made to a volume of 25 cc in 0 5 N HCl and 1 cc diluted to 10 cc with the dry organic solvent Addition of the amino acids in different millimolar concentrations permits identification of the peaks by height and integration

It will be noted that stock amino acid mixtures are stored in aqueous HCl solution and made to volume with the desired organic solvent immediately before use The excess alcoholic solution is discarded No alteration in the composition of the aqueous mixtures has been noted when they are stored at 3° for periods up to several months A progressive decrease in the amino acid content of mixtures of amino acids stored in the acidic alcoholic solutions has been observed Concomitantly with the decrease in free amino acid content of such solutions, the appearance has been noted of material which yields a fast moving zone on the column and gives a positive color reaction with ninhydrin The behavior on the column of such altered amino acid solutions has been simulated by the addition, to fresh amino acid solutions, of an amino acid ester, such as phenylalanine ethyl ester hydrochloride Some of the amino acids are esterified, therefore, upon prolonged standing in the acidic alcohol-water solvent mixtures The

[5] For quantitative work each pipette used was calibrated for delivery with the given organic solvent mixture

brief period of contact while the solution is being added to the column is insufficient to cause losses. Nearly quantitative recoveries are obtained even when an acidic solvent is employed in the development of the chromatogram (butanol-17 per cent 0.57 N HCl, Fig. 1).

In general, the amino acids are added to a column as their hydrochlorides in order to render the amino acids more readily soluble in the organic solvents. Of the six amino acids included in Fig. 1, all but tyrosine can be brought into solution in neutral butanol-water or butanol-benzyl alcohol-water. Chromatography of the neutral amino acid samples yields effluent curves indistinguishable from those obtained when the hydrochlorides of the amino acids are added to the column. The HCl, when included in the sample, is stripped away by the first few cm. of the starch. The fate of HCl can be visualized by incorporating a trace of methyl orange in the solvent. The HCl gives a zone which moves at about half the rate of valine and which fades out after traveling about 3 cm. as a result of gradual neutralization by the traces of basic groups present in the potato starch.

For the majority of the present experiments with protein hydrolysates, the proteins were hydrolyzed for 16 hours with 10 times their weight of boiling 6 N HCl. The most recent analyses have been carried out on hydrolysates prepared by dissolving the protein in a minimum volume of water or dilute HCl and adding 200 times the sample weight of 6 N HCl[6] twice distilled in glass. The mixture was refluxed for 16 hours on an oil bath, the oil level being kept below that of the contents of the flask. In both instances, the excess HCl was removed by repeated concentrations under reduced pressure. The hydrolysate was not filtered but was washed into a volumetric flask with small portions of water and made up to a volume corresponding to about 50 mg. of the original protein per cc. For chromatographic analysis, aliquots of this solution were diluted (0.5 cc. to 5 cc.) with the organic solvent and applied to the column in the manner outlined for the synthetic mixtures. The quantity of protein to which a given amount of hydrolysate corresponded was determined by micro-Kjeldahl nitrogen analyses on the protein and on the hydrolysate. The analyses indicated on the average about 2 per cent manipulative loss during the concentration procedure. A nitrogen analysis was also performed upon a solution obtained after 50 cc. of 6 N HCl, containing no protein, had been refluxed for 16 hours and concentrated to a volume of 5 cc. The nitrogen contributed by NH_3 in the HCl was negligible.

Collection of Effluent Fractions—In the early stages of this work, the

[6] Dr. C. F. Jacobsen has discussed with us his unpublished experiments which have demonstrated the advantages of carrying out the hydrolysis in relatively dilute solution with HCl which is as free as possible from heavy metal impurities. Humin formation is reduced under these conditions.

effluent fractions were collected manually The performance of a large number of experiments of the kind illustrated in Fig 1 became a practicable laboratory procedure only after the construction of a fully automatic fraction-collecting machine, drawings of which are given in Fig 2[7,8]

The machine is designed to collect the effluent from the chromatogram in successive fractions of known volume The fractions are accumulated in tubes held in the large circular test-tube rack which accommodates 80 test-tubes in each of four concentric circles The delivery tip of the chromatograph tube makes contact with the bottom of the enlarged part of the funnel (*A*) which is mounted in the phototube housing (*B*) The funnel is a Pyrex Gooch crucible holder 32 × 160 mm (Corning Glass Works, catalogue No 9480), the tip of which has been constricted Each drop falling from the tip of the funnel intercepts a light beam which is focused through a circular aperture $\frac{1}{8}$ inch in diameter upon a phototube (RCA No 921) The tip of the funnel must be mounted above the aperture at such a height that the emerging drops intercept the light beam just as they leave the tip If the tip is too low, hanging drops will register, if too high, the resulting free falling drop will pass through the light beam too rapidly to cause an interruption of the beam of sufficient duration to register the drop The height of the funnel can be adjusted by sliding it in the clamp (*C*) The glass shield (*D*) minimizes evaporation from the drop

The change in current of the phototube circuit occasioned by the falling drop is amplified through the relay (*E*) and fed into the automatic reset impulse counter (*F*) The phototube, lens, light source and relay system are available from the Langevin Corporation, 37 West 65th Street, New York (model PR-411-A), or from the Ripley Company, Inc , Deep River, Connecticut (modified 70 series, model No 8382) The housing and mountings were constructed of bakelite The impulse counter was purchased from the Eagle Signal Corporation, Moline, Illinois (type HZ-50A6, housing HN-84, wiring arrangment No 1) The impulse counter can be set to record any number from 1 to 400 drops After the preset number of drops has been registered, the counter resets itself to zero and turns on the motor (*G*) (110 volt, 60 cycle A C , 1/20 horse power, 1725 R P M , 10 1 gear reduction, Boston Gear Works, North Quincy, Massachusetts, catalogue No MB-5810-S) By means of a belt drive to the wheel (*H*), the motor turns the

[7] This machine was constructed with the aid of Mr Joseph Blum of the Instrument Shop of the Institute A photograph of the fraction collector is included in another report (10)

[8] A fraction collector based on this design is available commercially from the Technicon Company, 215 East 149th Street, New York 51 Convenient 50 tube aluminum racks with cellophane covers for handling the 18 × 150 mm test tubes are also available from this source

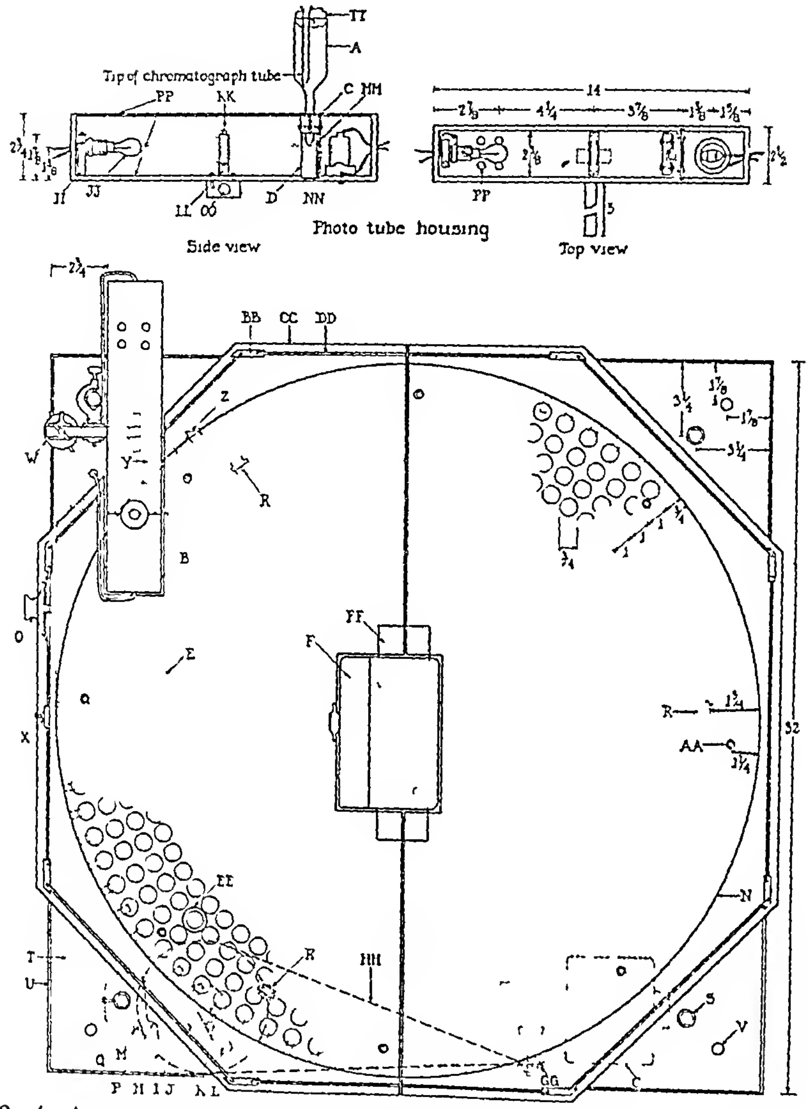

FIG 2, *A* Automatic fraction collector for chromatographic analysis, top view

FIGS 2, *A* AND *B* *A*, funnel, *B*, phototube housing (base and ends $\frac{1}{4}$ inch bakelite, sides 3/16 inch, top 3/32 inch, split into two sections at the funnel), *C*, split bakelite clamp for funnel ($\frac{3}{4} \times \frac{3}{4} \times 1\frac{1}{2}$ inches, 5/16 inch hole, thumb-screw adjustment), *D*, glass shield (1.8 cm tubing, 2 inches long), *E*, relay held to base of machine by brackets, *F*, impulse counter, *G*, motor, *H*, 6 inch diameter pulley, *I*, brass wheel, 2 inches in diameter, 3/16 inch thick, *J*, brass engaging pin (centered $\frac{5}{8}$ inch from center of shaft, $\frac{1}{4}$ inch high, $\frac{1}{8}$ inch in diameter, with freely turning collar 19/64 inch outer diameter), *K*, slots to accommodate engaging pin (5/16 inch wide, 5/16 inch high, 1 inch deep, centers 4.5° apart, directly under the holes of the rack), *L*, arm on pulley shaft to actuate micro switch, *M*, micro switch (type 132-R, brown top), *N*, test-tube rack with four concentric circles each possessing eighty $\frac{3}{4}$ inch holes (base $\frac{3}{8}$ inch bakelite, rack 3/32 inch), *O*, sensitivity dial for relay circuit, graduated 0 to 100 over 270°, under spring

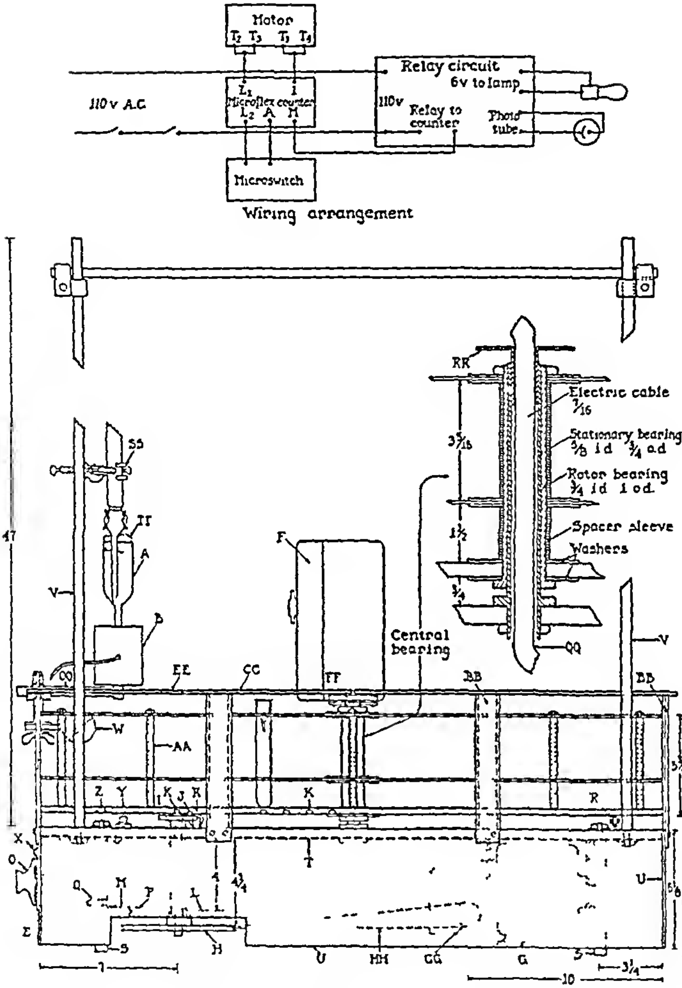

FIG 2, *B* Automatic fraction collector for chromatographic analysis, side view

tension to hold the knob in a set position, *P*, brass spring arm over micro switch, *Q*, aluminum plate to which microswitch is attached, held on leg of base by set screw, R, rollers ($\frac{3}{4}$ inch high, wheels $\frac{5}{8}$ inch diameter, $\frac{1}{4}$ inch wide), *S*, legs, $\frac{5}{8}$ inch aluminum rod turned down to 5/16 inch at the top, threaded for bolting through base, *T*, base plate, bakelite, 32 × 32 × $\frac{1}{2}$ inches, *U*, side panels, 3/32 inch bakelite (section cut out for 6 inch pulley on right side), *V*, $\frac{1}{2}$ inch Flexaframe rods, uprights turned down at the bottom to 5/16 inch, threaded for bolting through the base, with cross braces at the top, *W*, clamp, castaloy, Fisher catalogue No 5 764, *X*, on-off toggle switch, *Y*, toggle switch for automatic cut off, *Z*, beveled arm to throw automatic cut off switch, arm screwed to base of rack between slots, *AA*, one of eight spacers for rack, $\frac{1}{4}$ inch center rod, 5/16 inch spacing sleeves, *BB*, slotted brass supports for side panels of the cover, *CC*,

wheel (I) which is adjacent to the rack The upright pin (J) on this wheel engages one of the slots (K) in the base of the circular test-tube rack and moves the rack one-eightieth of a turn A new tube is thus brought under the column The arm (L) on the shaft makes contact with the micro switch (M) and turns off the motor after the wheels (H and I) have made one revolution Since the counter mechanism requires that the load circuit be closed for nearly 1 second to allow time for complete resetting, it is necessary for the gear ratio to give not more than one revolution of H per second The moving parts of the rack are machined from brass The rack revolves on a sleeve bearing at the center and is supported by three symmetrically placed rollers (R) mounted on the base

The fraction collector is designed for continuous duty The counter and the relay can be replaced readily in case of failure An extra one of each of these items should be kept on hand as replacement parts The relay is mounted open to the air (not in a closed housing) under the base of the machine to avoid overheating The light bulb is changed routinely after each month of continuous duty In the installation of the photoelectric counter, it is necessary to keep the low resistance leads to the phototube short enough to bring the circuit into counting balance when most of the variable resistance (O) is in the circuit The relay should close when the sensitivity dial (O) is turned to about 70 Accurate focusing of the light beam on the aperture is essential for this result and the aperture in the diaphragm may have to be enlarged if the light intensity striking the phototube is too low The adjustment of the tip should be such that drops still register when the sensitivity dial is near zero For maximum stability during operation, the dial is set about midway between the point at which the counter almost fails to register drops and the dial setting at which the counter circuit is permanently closed The relay is designed to give an impulse of about 0 5 second duration to the counter upon interruption of the light beam for 0 01 second The maximum counting rate with this machine is, therefore, about 2 drops per second

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⅛ inch Lucite cover, split in center, cut out around phototube housing, *DD*, 1/16 inch Lucite side panels, 7½ inches high, *EE*, 1¼ inch hole in cover, centered over third row of rack, *FF*, center supports for cover, attached to base of counter, *GG*, 1 inch diameter pulley, *HH*, 3/16 inch leather belt, *II*, split bakelite clamp for lamp socket, *JJ*, lamp, General Electric 87, 6 to 8 volts, 15 candle power, mounted with the filament vertical, *KK*, lens, 1½ inches in diameter, 2 inch focal length, *LL*, grooved lens holder of bakelite, providing a firm grip on the lens, *MM*, removable diaphragm of 3/32 inch bakelite, supporting clamp *C*, sliding into grooves in side walls of housing, with ⅛ inch diameter aperture for light beam, *NN*, phototube, RCA 921, *OO*, ½ × 5 inch supporting rod for housing, *PP*, ventilating holes in housing, *QQ*, six wire electric cable to counter, *RR*, base plate for counter, with hollow central shaft fitting tightly into stationary bearing, *SS*, clamp, castaloy, Fisher catalogue No 5-743, *TT*, cotton packing around stem of chromatograph tube

In the design of the fraction collector, operation on the drop-counting principle was chosen to provide rigorous control of the sample size Experience with the machine has demonstrated that, with a dependable drop counter of the type recommended, considerable convenience and accuracy are afforded by this method of operation For chromatographic work in general, fraction collectors operated on a time basis would probably not give as uniform fraction size, since flow rates through a column are seldom strictly constant, even when constant pressure devices are employed

The base of the machine and the circular rack should be made of materials which do not warp and are not damaged by organic solvents or dilute acids The rack should be of material which will not scratch photometer tubes Bakelite has been used in the present instrument, although investigations performed recently and to be described in a forthcoming paper have revealed that this material suffers from the disadvantage that it contains ammonia or other volatile nitrogenous compounds In chromatrographic experiments with the amino acids not included in the present work, acidic solvents, such as 2 1 n-propanol-0 5 N HCl, have proved extremely useful Such solvents absorb ammonia very readily Since as little as 0 1 γ of NH_3 per cc is detected by the ninhydrin method, the NH_3 content of the bakelite has been troublesome It has been possible to obtain satisfactory operation by painting the bakelite surfaces of the machine and of the phototube housing with a 2 per cent alcoholic solution of citric acid Sheets with a dull finish take this treatment better than polished bakelite Nevertheless, in building the fraction collector, it would be preferable to use materials which do not liberate ammonia Provided the manufacturers cannot furnish bakelite meeting these specifications, a possibility currently being investigated, other materials might be substituted Any material employed should first be tested by suspending a sample of it over a few cc of 2 1 propanol-0 5 N HCl in a closed vessel for 24 hours Analysis of neutralized 0 5 cc aliquots of the solvent by the ninhydrin method should reveal the presence in the material of any volatile nitrogenous compounds which might be a source of interference The Lucite cover is not resistant to butanol or benzyl alcohol and it may prove preferable to make covers from other materials A sliding addition to the cover can also be made to fill in the space beneath the phototube housing

In a chromatographic experiment, the starch column, to which the sample of amino acids has already been added, is mounted on the fraction collector The solvent reservoir is attached, and the requisite air pressure applied The stop-cock on the reservoir is clamped firmly in position to prevent it from loosening under pressure With columns 0 9 cm in diameter, 0 5 cc fractions are collected In general, in order to realize the full resolving power of the column, the size of the fractions collected should be

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small enough to yield at least ten points on each amino acid peak. The number of drops required for a given volume is readily determined from the weight of the liquid delivered in 10 drops from the tip, together with the density of the solvent. For small volumes, a tip which delivers 20 to 40 drops per 0.5 cc. is convenient. The size of drop delivered is not significantly changed by a 2-fold alteration in the rate of flow. The number of drops per cc. from a given tip is constant to better than 1 per cent for a given solvent over a temperature range of several degrees and is unaffected by dilute concentrations of solute in the effluent. The drop size can be increased to a maximum of about 20 drops per cc. with a fairly blunt tip of 8 mm. outer diameter. If water is being used, rather than an organic solvent mixture, a beveled tip or a constricted tip of wider bore may be required for even flow.

The machine is designed to hold 80 lipless soft glass test-tubes, 18 × 150 mm. (Arthur H. Thomas Company, catalogue No. 9446). For analytical experiments with columns 0.9 cm. in diameter, much time is saved by employing tubes that have been calibrated for use in the spectrophotometer (11). Since the whole fraction is analyzed, evaporation of some of the solvent introduces no error. If larger columns are being run for preparative purposes, uncalibrated tubes are employed and aliquots pipetted for analysis. In this case, evaporation can frequently be neglected, since with butanol it amounts to only about 50 mg. per tube in 18 hours. If desired, evaporation from the tube can be reduced almost to zero by placing a small funnel in each tube, as illustrated in the receiver in Fig. 2, *B*. The funnels can be made from 15 × 125 mm. Pyrex test-tubes with rims. In the collection of large fractions, the splashing which frequently occurs as the drops strike the funnels can be eliminated by placing a small pad of glass wool in each funnel.

The tubes are permanently numbered with a serial number and a set number and stored in sets of 100 to 200 tubes in soft aluminum racks,[8] protected from dust by covers. The filled tubes are removed from the fraction collector each day and replaced by empty receivers. The fractions are stoppered with corks until ready for analysis. A typical run for the six peaks shown in Fig. 1 requires continuous operation of the machine for about 4 days. Under these conditions the automatic cut-off arm (*Z*) is removed from the base of the rack. If it is desired to have the machine turn off after filling a prescribed number of tubes, this arm can be placed in position. As many as four columns can be run on one machine at the same time. For example, a second column can be mounted on the right side over the second or third row of receivers and the pressure adjusted to give about the same rate of flow as that of the column feeding into the photoelectric counter. The tip, with cotton packing around the stem, is protected by

a glass shield fitting into an opening in the cover (*EE*, Fig 2) For analytical experiments with columns 0 9 cm in diameter, this arrangement can be satisfactory, since the exact fraction size does not enter into the integration of the curves However, if the fraction size is not accurately known, the estimate of the position of a peak in terms of cc on the abscissa of the effluent curve may be in error If the second row experiment is a duplicate of one that has been run on the counter, the peaks can usually be identified by cross-reference to the sequence of peaks in the first determination Columns on the inner rows can also be used for exploratory qualitative experiments

Analysis of Effluent Fractions—When columns 0 9 cm in diameter are employed, entire 0 5 cc effluent fractions are analyzed by the photometric ninhydrin method outlined in the following paper (11) With larger columns, 0 1 to 0 5 cc aliquots of the effluent fractions are pipetted for analysis The convenience of eliminating the pipetting step points to the desirability of employing columns 0 9 cm in diameter for experiments being run for analytical purposes

In the plotting and integration of the curves, it is important that the base-line be correctly chosen With each set of 50 fractions taken for analysis, six to eight fractions well ahead of or behind the peaks should be included These fractions determine the blank reading of the effluent from the column The positive fractions are read against the average tube of the blank series as zero

The curves are integrated by the addition of the analytical values for the points on a given peak In determining the mg of sample placed on the column, calibration factors for the delivery of the pipettes with aqueous and alcoholic solutions are included (*cf* (11), Table V)

When two or more amino acids are incompletely separated from one another, as in the case of tyrosine and valine in butanol-benzyl alcohol (Fig 1), the amounts of each can be calculated if the overlap does not extend as far as the peak points The calculation depends upon two experimentally observed facts, namely, (*a*) the height of a given peak is proportional to the amount of amino acid represented by the peak, and (*b*) for a given column, the height of a peak of unit area decreases approximately linearly with increasing effluent volumes In general, the calculation is made as follows

A_1, A_2, and A_3 = quantities in micromoles of amino acid in the first, second, and third peaks

P_1, P_2, and P_3 = corrected height of each peak in millimolar concentration from Tables I and III of the following paper (11)

V_1, V_2, and V_3 = effluent volumes at which the peaks emerge

F_1, F_2, and F_3 = color yields for amino acids A_1, A_2, and A_3

T = micromoles (leucine equivalents) obtained by integration of the combined peaks, as in Table V (11), not corrected for color yield

$$A_1 = \frac{P_1 T}{F_1\left(P_1 + \frac{V_2}{V_1}P_2 + \frac{V_3}{V_1}P_3\right)}$$

$$A_2 = \frac{\frac{V_2}{V_1}P_2 T}{F_2\left(P_1 + \frac{V_2}{V_1}P_2 + \frac{V_3}{V_1}P_3\right)}$$

$$A_3 = \frac{\frac{V_3}{V_1}P_3 T}{F_3\left(P_1 + \frac{V_2}{V_1}P_2 + \frac{V_3}{V_1}P_3\right)}$$

The recoveries of tyrosine and valine summarized in Table I have been calculated by this method by using only the terms involving P_1 and P_2 for a double peak The calculation gives results within ±5 per cent of the theoretical recoveries in this instance In order to apply these equations, it is necessary to have several analytical points near the peak of the curve The recoveries are less satisfactory when the peaks are rising so rapidly that the maximum concentrations are not well defined It is also necessary that the fraction representing the peak of each curve should contain only one amino acid The decision as to whether the peaks are sufficiently separated to permit valid calculation can generally be made by inspection of the curves With unknown mixtures, the applicability of the calculation should be checked by an experiment with a known mixture containing tyrosine and valine in approximately the same proportions found in the unknown The use of partially separated peaks for analytical purposes is to be avoided whenever possible by the choice of a more favorable solvent mixture The calculations given above have proved useful, however, for preliminary approximations, as well as in securing the data on tyrosine and valine given in Tables I, II, and III

In some instances with larger chromatograms, it is convenient to be able to spot-test the fractions with ninhydrin-impregnated paper in order to locate the positions of major peaks and valleys Strips of filter paper can be impregnated with a solution of 100 mg of ninhydrin in 10 cc of n-propanol and 20 cc of 0 2 M citrate buffer, pH 5 The air-dried paper can be stored for a week or more in the dark without significant deterioration After the application of a small drop from each fraction, the paper is warmed to 80° for development of the blue color With butanol-benzyl alcohol, it is desirable to place a drop of water on the paper before adding the organic solvent

Solvent Mixtures[9]—The solvent which has proved most useful for the separation of the first six amino acids (Fig 1) contains n-butanol, benzyl alcohol, and water Consden, Gordon, and Martin suggested the use of this combination of alcohols in paper chromatography (4) On starch, the mixture possesses the advantage, in comparison with butanol alone, of moving phenylalanine well ahead of leucine and isoleucine (Fig 1) With this solvent, however, recoveries of methionine have been low, occasionally by as much as 50 per cent Oxidation of methionine by traces of peroxides present in the benzyl alcohol appears to be responsible for this effect[10] Quantitative recoveries of methionine are obtained when butanol is used alone The recoveries can also be made quantitative in the butanol-benzyl alcohol solvent by the inclusion of 0 5 per cent thiodiglycol (redistilled Kromfax solvent) The solvent mixture which has been adopted for the chromatographic analysis of the first six amino acids (Fig 1) is made up of 500 cc of n-butanol, 500 cc of benzyl alcohol, 144 cc of water, and 5 cc of thiodiglycol If the thiodiglycol is not added, the water content is reduced to 142 cc

At 25°, the above solvent is slightly undersaturated with respect to water It is essential that all solvents meet this specification If saturated solvents are used, slight changes in temperature may induce the separation of free droplets of water in the solvent above the chromatogram The collection of excess water on the top of the starch column can markedly distort the amino acid peaks The amount of water in the solvent also requires careful control in order to effect optimum separation of tyrosine and valine If the water content of the above mixture is decreased to 138 cc, the tyrosine and valine peaks are so close together that there is no evidence of a dip or valley in the curve The curve in Fig 1 was obtained before this factor was fully appreciated and the result shown is therefore marginal The increase of the ratio of benzyl alcohol to butanol to 55 45 did not permit a wider variation in the amount of water in the solvent The relative positions of the other amino acid peaks are not affected by similar small variations in the water content For experiments with methionine, the solvent should be used within 2 weeks after the addition of the thiodiglycol

Butanol saturated with water contains about 170 cc of water per liter For the chromatogram shown in Fig 1, *B*, an undersaturated solvent is prepared by diluting 150 cc of water to 1 liter with n-butanol The acidic solvent for Fig 1, *C*, was prepared from 170 cc of 0 57 N HCl made up to 1 liter with butanol Since the presence of HCl increases the solubility of

[9] The organic solvents employed in this work have been of analytical reagent grade Distillation prior to use has been found unnecessary

[10] The peroxide content of different lots of benzyl alcohol varies as judged by the KI test Samples giving a strongly positive reaction are not used Even when the KI test is essentially negative, thiodiglycol must be included in the solvent

water in the alcohol, this solvent is still slightly undersaturated The curve with the butanol-HCl mixture has been included in Fig 1 for comparison This solvent is unsatisfactory for many purposes, since the recoveries of amino acids may run somewhat low because of esterification

The solvents described above are only three examples from the variety of mixtures that can be tried with starch columns, depending upon the objectives of the experiment Further work will be reported on the use of solvents of higher water content for the fractionation of the slower moving amino acids such as alanine and glycine, and the acidic and basic amino acids These components are still on the column at the end of the experiments described in the present communication

DISCUSSION

Variations in Starch—The potato starch manufactured by Morningstar Nicol, Inc , which was used in the present experiments, contained 0 3 per cent ash (as sulfate), 0 05 per cent nitrogen, and 12 to 20 per cent moisture The granules varied in size from 0 01 mm to 0 06 mm with the diameter of the average particle being about 0 03 mm It has been noted that the starch is capable of neutralizing small amounts of HCl When 20 gm were suspended in 50 cc of 0 01 N HCl and an aliquot of the supernatant was titrated, it was found that 1 5 cc of 0 01 N HCl were neutralized per gm of starch Small quantities of ash and other impurities are extracted from the starch by the butanol and butanol-benzyl alcohol solvents This material does not affect the analytical experiments with starch chromatograms For isolation work, it may prove desirable to wash the starch four to six times with 6 volumes of distilled water Before it is dried, the water-washed starch should be washed with ethanol in order to facilitate the production of a finely divided air-dried powder For analytical experiments, the starch is used as it comes from the manufacturer, as further treatment has not served to improve its resolving power Samples of starch washed and dried in the laboratory have actually not given quite as good column performance as the untreated commercial material

Experiments have also been carried out with a sample of potato starch purchased from Messrs Gordon Slater, Ltd , Manchester, England, which presumably corresponds to the material employed by Synge (6) The ash content was less than 0 1 per cent and the nitrogen content was 0 05 per cent The particle size was significantly larger than that noted above The average granule had a diameter of about 0 04 mm When exposed to a saturated atmosphere of water or butanol containing 15 per cent water in a desiccator at 25°, both the Morningstar Nicol and the Gordon Slater preparations adsorbed 50 per cent of their dry weight of water or water plus

a small amount of butanol This value is higher than the value of 35 per cent reported by Synge (6)

In chromatographic work, the reproducibility of the adsorbent is a key factor in determining the usefulness of a given fractionation procedure In the present experiments with starch, it has been found that almost identical results can be obtained with different batches of potato starch from different manufacturers when measures are taken to compensate for variations in trace metal contamination and in particle size

The effect of small amounts of amino acid complexes of metal ions was observed in early experiments in which the first peak through the column was markedly distorted (10) It was found that this difficulty could be completely overcome by removing the interfering ions with 8-hydroxyquinoline When a chromatogram was run with the Gordon Slater starch without treating it with hydroxyquinoline, the effect of the metal ions was not limited to the first peak, but rendered the whole chromatogram completely unsatisfactory In butanol-benzyl alcohol the first three components emerged as one broad zone with a recovery of only 55 per cent Prior hydroxyquinoline treatment yielded a curve almost indistinguishable from that in Fig 1 The resolution was nearly as good and the peaks were in the same positions With this starch, however, it was necessary to use 4 times as much hydroxyquinoline as that prescribed for the Morningstar Nicol starch Therefore, if poor resolution is obtained with a given starch, an increase in the quantity of hydroxyquinoline may be tried If possible, it is preferable to procure a sample of starch which requires only the minimum treatment with hydroxyquinoline The metal effect is not correlated with the total ash of the starch The interference is due to a minor inorganic component and it is not necessary to carry the hydroxyquinoline extraction to the point of completion To continue the extraction to the point where no more colored complexes are eluted may require about 10 times the amount of hydroxyquinoline necessary to yield optimum amino acid resolution

Columns packed with the Gordon Slater sample of starch flowed at about twice the optimum rate when poured originally with 30 per cent water content The increase in flow rate correlates with the observation on the larger granule size The satisfactory results cited above were obtained on columns poured with an initial 20 per cent water content to compensate for this difference

Starches other than potato starch have been studied Commercial cornstarch, after purification by acid-alcohol extraction to remove nitrogenous impurities and lipides, gave effluent curves which were qualitatively the same as those obtained with potato starch The resolving power was much inferior and it was not determined whether improvements in the packing

procedure could be made Rice starch, with its much smaller particle size, gave flow rates that were inconveniently slow Canna starch,[11] which posesses the largest granule size among the common starches, gave less uniform column flow when tested with a colored sample zone On the basis of purity, availability, and functional tests, potato starch appears to be preferable to other starches for chromatographic work with amino acids

Techniques of Column Packing—The degree of resolution of phenylalanine, leucine, and isoleucine in the butanol-benzyl alcohol solvent mixture has been used as a test in studies on the effect of variations in the packing procedure With a poor column, the peaks are in the same positions but are lower and the spreading of the zones tends to fill in the valleys The use of electric vibrators during packing, rotation of the column during pouring, or continuous agitation of the slurry during its introduction appeared to have little influence on the results After the accumulation of observations on a large series of columns of varying efficiencies, the most important point in the packing procedure proved to be the water content of the starch at the time the column was prepared Control of the water content affects the uniformity of the packing in two ways First, the density of the starch granule decreases with increasing hydration The density of the particles at 20 to 30 per cent water content is such that they settle very slowly and evenly in dry butanol, but the density difference is not so small that the granules tend to "float" in the solvent The observation of a sharp settling line moving up the chromatograph tube is evidence for a satisfactory density difference Secondly, the swelling of the starch granules from about 30 per cent water content to 50 per cent, which occurs after the column has settled to constant height and while it is equilibrating with the wet organic solvent, creates an evenly distributed internal pressure capable of reducing the intergranule interstices The high efficiencies of these columns result in large part from the particular properties of the starch granule which make possible a packing procedure of the type employed

As the initial water content of the starch during the pouring of the column is decreased below 20 to 30 per cent, no further significant increase in column efficiency has been obtained The packing procedure outlined in the experimental section routinely gives columns which are essentially identical in flow characteristics and resolving power This generalization holds for columns up to 8 cm in diameter, the characteristics of which can be accurately predicted from runs on columns 0 9 cm in diameter

Rate of Flow—Decrease of the rate of flow below that prescribed in the experimental section has not yielded improved resolution Doubling the

[11] We are indebted to Mr C V Caesar of the Stein, Hall and Company, Inc, New York, and to Dr C O Beckmann for the samples of canna starch.

flow rate by increasing the pressure has caused about a 10 per cent decrease in the heights of the peaks and corresponding spreading into the valleys This increased flow rate requires the use of a piston assembly of the type described by Claesson (12) to avoid contact between the solvent and the air at the higher pressure The solvent was added from a 100 cc glass syringe attached to the top of the column The syringe was enclosed in a brass container fitted to the upper part of the chromatograph tube through a rubber stopper The maximum pressure of 15 cm, which has been adopted for the present experiments, is convenient from the standpoint of operation and gives optimum resolution

Variations in Amino Acid Sample—Successful fractionation of a mixture of amino acids on a starch column depends upon not overloading the column The quantity of material that can be handled will vary with the composition of the mixture and the objective of the experiment With the protein hydrolysates studied in the present experiments, the maximum total load of 2 5 mg on a butanol-benzyl alcohol column causes a narrow white zone to form at the top of the translucent column This zone is associated with the liberation of small amounts of water which cannot be absorbed by the slightly undersaturated solvent If the amino acid load is doubled, the amount of water liberated may be sufficient to cause visible streaks to spread down the walls of the tube Under these conditions, the peaks emerging in the effluent are broadened and resolution is inferior The glycine, glutamic acid, lysine, ammonium chloride, and other very hydrophilic components of the mixture contribute to the limiting load in this case The presence of significant percentages of NaCl, or other inorganic salts, may have the same effect

The loading of phenylalanine, leucine, and isoleucine can be increased in the present experiments, if these constituents are the only components of the mixture If the objective of the experiment is the determination of methionine, which gives a peak well separated from the rest, the load can be increased without regard to some reduction in resolving power Also, if the objective is the isolation of components from the effluent of a large column, columns can be operated at a higher capacity and the overlapping zones discarded in working up the fractions

Identification of Amino Acid Peaks—The effluent volume at which a given amino acid peak emerges from the column has been established in a series of experiments with simple and complex known mixtures In experiments with relatively simple unknown mixtures, such as acid hydrolysates of purified proteins, the considerations described below make it possible to identify the familiar amino acid peaks with a relatively high degree of certainty It should be stated at the outset, however, that in chromatographic work with unknown mixtures the only unequivocal method for the

qualitative identification of the component or components in a peak is through isolation of the material in sufficient quantity to permit its characterization by independent microchemical methods The fact that the starch column can be scaled up to 8 cm or more in diameter indicates that this approach is feasible Further work is required on techniques for the isolation of components from the effluent on a preparative scale

Identification of the peaks is facilitated by the fact that the effluent volume for a given amino acid has been found to be constant to within ±5 per cent, depending upon the nature of the solvent and the weight of starch used in the preparation of the chromatogram. The position of an individual amino acid has not been influenced by the presence of other amino acid components in the mixture being fractionated Leucine, for example, emerges at the same position when added in a synthetic mixture as when added alone In the graphical presentation of the results, it has been convenient to measure the effluent volume from the time the sample is placed upon the column, without subtracting the initial column volume Small variations in the positions of the effluent peaks are thereby introduced as a result of differences in the tightness of the column packing The variations from this source are negligible in the present experiments

Comparison of the knowns and unknowns with respect to the general sequence of the peaks and their precise positions relative to one another is of importance (Figs 1, 3, and 4) The position of the leucine peak relative to the neighboring phenylalanine and isoleucine peaks is even more reproducible than the absolute effluent volume The exact position of a peak on the abscissa may change slightly, for example, with small variations in the composition of the solvent The relative positions of two peaks are seldom similarly sensitive An exception is the case of tyrosine and valine discussed earlier

The positions of the peaks obtained in the analysis of an unknown mixture can be checked by the addition of one or more known amino acids to the sample prior to analysis The corresponding peaks on the effluent curve should rise without loss of symmetry, and the added amount of amino acid should be recovered quantitatively In some cases, information on the identity of a peak can be obtained by specific color reactions In the tyrosine range, where frequently only every second fraction is used for ninhydrin analysis, the remaining fractions containing tyrosine can be pooled, concentrated to dryness, and tested with Millon's reagent The probability of correct identification can also be greatly increased by demonstrating that the peak from the unknown appears at the correct position when chromatographed with two or more different solvents

When working with mixtures more complex than the usual protein hydrolysate, the problems of identification are increased Conclusions

based upon chromatographic data alone should be made with caution, particularly in the case when the mixture has not been subjected to hydrolysis In such instances, in the absence of additional information, it cannot be assumed that a peak in the effluent curve occurring in the phenylalanine range, for example, is phenylalanine Nevertheless, information useful as a basis for further work may be obtained Care in the examination of each peak for its absolute position, its position relative to other peaks, and any unusual degree of asymmetry may reveal the presence of unsuspected components If a peak should occur in a portion of the curve normally unoccupied by any of the common amino acids, it is possible to state definitely that it is not one of these substances Similarly, the absence of an amino acid can be unequivocally demonstrated within the accuracy of the ninhydrin method It must be borne in mind that the ninhydrin method of analysis is sensitive only to compounds containing amino groups A single symmetrical peak on the effluent curve does not exclude the presence of ninhydrin-negative compounds with similar rates of travel on the chromatogram

Among the possible fast moving components other than those shown in Fig 1 is tryptophan which emerges near to phenylalanine in the three solvent mixtures In the butanol-benzyl alcohol solvent, this amino acid is readily detected in the valley between phenylalanine and leucine, and in Fig 4, *A* would yield a peak at an effluent volume of 38 5 cc If the separation of phenylalanine, tryptophan, and leucine is required, experiments have indicated that they can be completely differentiated by rechromatographing the mixture on an aqueous 0 1 N HCl column (10) With acid hydrolysates of proteins, the problem seldom arises, since tryptophan is usually decomposed during the hydrolytic process Other possible amino acid components in the leucine-valine range include dibromo- and diiodotyrosine The latter emerges at an effluent volume of 16 cc (Fig 4, *A*) The presence of peptides of course, would introduce many possible additional components

There are no detectable differences in the rates of travel of D-, L-, and DL-amino acids on the starch column This point has been checked with the L and DL forms of all of the amino acids covered by the present experiments, except tyrosine

The positions of the amino acid peaks are also of significance in the theoretical interpretation of the action of the starch column It has been pointed out that the rates of travel of the amino acids on the column do not correspond in all cases to the rates to be expected from the liquid-liquid distribution theory (10) Further studies on the acidic and basic amino acids have revealed similar discrepancies The available data suggest that the underlying principles governing the operation of the starch column are

adequately covered by the currently accepted definition of the chromatographic process. The term "liquid-liquid (partition) chromatography" does not appear to be applicable to the starch column.

Quantitative Analysis of Synthetic Mixtures of Amino Acids—The results obtained in a series of chromatograms performed on synthetic mixtures containing the eighteen amino acids most commonly found in protein hydrolysates are summarized in Table I. Recoveries on columns 1.9 cm. and 0.9 cm. in diameter have been included. The results indicate that in work with protein hydrolysates, an individual determination of a component present to the extent of 3 per cent or more of the protein is seldom in error

Table I

Recoveries of Amino Acids from Mixtures Containing Nineteen Components

Solvent, 1:1:0.288 *n*-butanol-benzyl alcohol-water.

Mixture	Per cent recovery					
	Phenylalanine	Leucine	Isoleucine	Methionine	Tyrosine	Valine
Synthetic β-lactoglobulin hydrolysate*	101.4	101.0	103.5		97.9	102.0
	97.3	100.4	104.3		99.0	99.8
	98.6	104.5	106.8		101.8	103.0
Synthetic bovine serum albumin hydrolysate†	103.5	102.8	100.2		105.3	98.3
	103.6	101.0	102.0		106.4	96.7
	101.8	101.6	103.6		100.6	99.0
	98.4	102.0	100.0	100.0‡	101.1	100.1
	99.4	101.0	102.8	100.6‡	102.7	100.8
Average	100.5	101.7	102.9	100.3	101.8	100.0

* Corresponding in composition to an acid hydrolysate of β-lactoglobulin (Brand *et al.* (7)). Tryptophan was omitted.

† Corresponding in composition to an acid hydrolysate of bovine serum albumin (Brand (13)). Tryptophan was omitted.

‡ The solvent contained thiodiglycol. The amount of methionine present was increased to 6 times that reported for bovine serum albumin.

by as much as 5 per cent. The averages of several determinations have given an accuracy of ±3 per cent.

Composition of Hydrolysates of β-Lactoglobulin—The sample of β-lactoglobulin used in these experiments was prepared in the laboratory of the late Dr. Max Bergmann by Dr. G. Haugaard and was one of two samples recently analyzed by Brand and coworkers (7). The figure of Brand *et al.* for the nitrogen content of the ash- and moisture-free protein, 15.6 per cent, was confirmed and was employed as a basis for calculations in the present work. This protein offered opportunity for comparison of the analytical results obtained by chromatography on starch with those obtained by other methods.

The effluent curve from a sample of the hydrolysate showed no unexpected peaks (Fig 3) The positions of the six peaks present corresponded

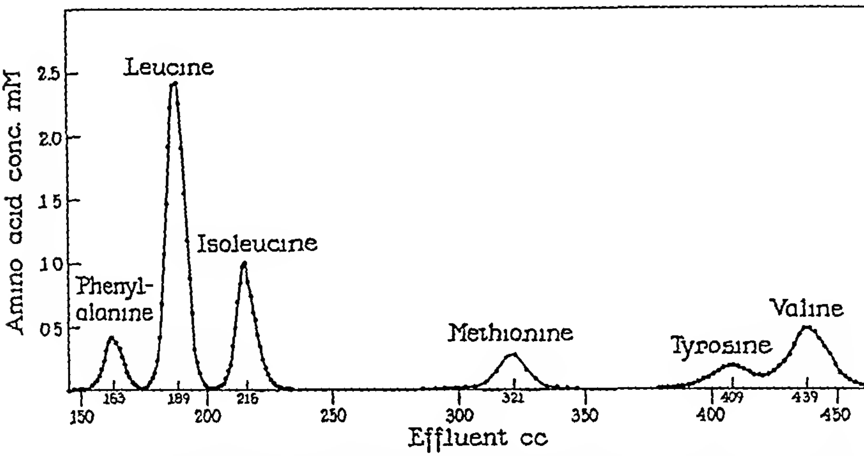

Fig 3 Chromatographic analysis of a hydrolysate of β lactoglobulin Solvent, 1 1 0 288 *n*-butanol-benzyl alcohol-water (without thiodiglycol) Column, 52 gm of starch (anhydrous), diameter, 1 9 cm , height, 30 cm Sample, about 20 mg of amino acids

Table II

Amino Acid Composition of Hydrolysates of β Lactoglobulin

The numbers in parentheses refer to the bibliography

Method of determination	Hydrolysate No	Amino acid, gm per 100 gm protein				
		Phenylalanine	Leucine	Isoleucine	Tyrosine	Valine
Chromatography on starch	1	3 74	15 4	5 81	3 58	5 71
	2	3 82	15 7	6 04	3 81	5 61
	2	3 77	15 5	5 74	3 52	5 55
Average		3 78	15 5	5 86	3 64	5 62
Isotope dilution			15 7(14)			
Microbiological		3 54(7)	15 4(15)	8 4(7)		5 8(7)
		4 3(16)	15 3(16)	7 0(16)		5 5(16)
				8 7(17)		
				6 1(18)		
Chromatography on silica gel		4 2(3)				5 8(3)
Solubility product			15 9(19)			
Photometric					3 78(7)	

to those of recognized components of the protein When known amounts of phenylalanine and isoleucine were added to the hydrolysate, the peaks

assigned to these components rose accordingly to give recoveries of 99 and 98 per cent, respectively, for the added quantities The curve was comparable to that obtained with the synthetic control (Fig 1, *A*) The quantitative values obtained by integration of the curves are given in Table II

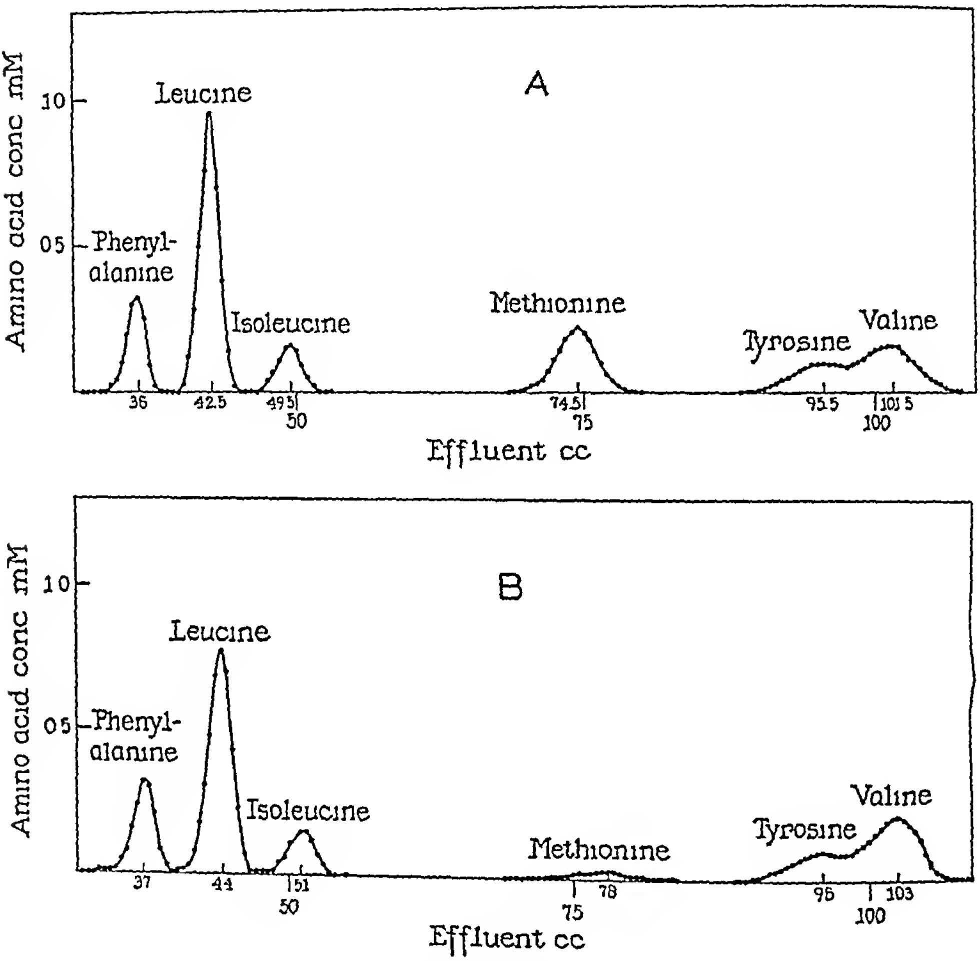

Fig 4 Chromatographic analyses of a hydrolysate of bovine serum albumin and a synthetic mixture of similar composition Solvent, 1 1 0 288 *n* butanol-benzyl alcohol water, containing 0 5 per cent thiodiglycol *A*, synthetic mixture corresponding to an acid hydrolysate of bovine serum albumin with increased methionine content Column, 13 4 gm of starch (anhydrous), diameter, about 0 9 cm , height, 31 cm Sample, about 2 5 mg of synthetic mixture *B*, hydrolysate of bovine serum albumin Column, 13 4 gm of starch (anhydrous), diameter, about 0 9 cm , height, 33 cm Sample, corresponding to about 2 5 mg of protein

The results are in reasonable agreement with the values obtained by other methods, except in the case of isoleucine for which the chromatographic value was more than 20 per cent below the earlier microbiological values The reason for the high results (7, 16, 17) has subsequently been ascertained

and the most recent value of 6 1 per cent obtained by Smith and Greene (18) is close to the chromatographic figure of 5 86 per cent Since the β-lactoglobulin experiments were run before thiodiglycol was incorporated into the solvent, quantitative methionine values were not obtained

Composition of Hydrolysates of Bovine Serum Albumin—The sample of protein analyzed was obtained through the kind cooperation of Dr Erwin Brand, and was the same preparation (Armour, lot No 18) which was analyzed in his laboratory The nitrogen content of the ash- and moisture-free protein, 16 07 per cent, given by Brand (13), was confirmed An

TABLE III

Amino Acid Composition of Hydrolysates of Bovine Serum Albumin

Method of determination	Hydrolysate No	Amino acid gm per 100 gm protein					
		Phenylalanine	Leucine	Isoleucine	Methionine	Tyrosine	Valine
Chromatography on starch	1	6 96	12 4	2 65		5 15	6 04
	1	6 42	12 4	2 67		4 76	5 86
	1	6 56	12 6	2 41		4 85	5 92
	2	6 39	11 9	2 52		4 96	5 70
	2					5 30	5 69
	3	6 56	12 0	2 74	0 92	5 18	5 95
	3	6 72	12 3	2 68	0 92	5 24	6 27
Average		6 60	12 3	2 61	0 92	5 06	5 92
Other methods		6 2*	13 7*	2 9*	0 81†	5 49‡ 5 53§	6 5*

* Microbiological assay (13, 20)
† Iodometric determination (13, 20)
‡ Photometric determination (13, 20)
§ Isotope dilution method (21)

effluent curve on an acid hydrolysate of bovine serum albumin is given in Fig 4 Integration of the peaks yielded the results given in Table III In this case the chromatographic values for leucine, isoleucine, tyrosine, and valine are all about 10 per cent below the values given by Brand (13, 20) Phenylalanine runs about 6 per cent higher and the methionine value is essentially a check However, the methionine peak was far too low for accurate integration With a peak as low as that of methionine in this case, an error of 0 005 in the optical density reading for the base-line of the peak could cause an error of 15 per cent in the recovery For amino acids present in small amounts, the accuracy can be increased by placing a larger sample on the column For tyrosine, the value of Brand and coworkers

(20) checks well the figure of Shemin (21) who employed the isotope dilution method In the latter case, however, a different lot of serum albumin was employed The uniformity of the discrepancy between some of the earlier results and those reported here stimulated a diligent search for systematic errors in the chromatographic procedure Three different hydrolysates were analyzed with concordant results The nitrogen content of the hydrolysates was determined by the same micro-Kjeldahl procedure used in the analysis of the protein The synthetic mixtures gave excellent recoveries (Table I) There appears to be no systematic explanation for the differences

SUMMARY

A procedure for the quantitative chromatographic separation of phenylalanine, leucine, isoleucine, methionine, tyrosine, and valine has been developed The amino acid mixture is fractionated on a column packed with potato starch The solvent which has been used in most of the experiments is 1 1 0 288 *n*-butanol-benzyl alcohol-water containing about 0 5 per cent thiodiglycol

Photometric ninhydrin analyses are performed on small increments of the effluent solution to permit the construction of effluent concentration curves which reveal in detail the changes in composition of the eluate With columns 0 9 cm in diameter and 30 cm in height, integration of the resulting peaks gives amino acid recoveries accurate to 3 to 5 per cent in individual determinations on 0 1 mg quantities of a component The average of several experiments gives recoveries to within ±3 per cent on synthetic mixtures of nineteen components corresponding in composition to protein hydrolysates Experiments have been carried out on the determination of the amino acid composition of acid hydrolysates of samples of bovine serum albumin and β-lactoglobulin

An automatic fraction-collecting machine is described for the collection of the large number of small effluent fractions required in this type of chromatography The techniques for measurment of the shape and position of the emerging peaks have permitted careful comparison of different samples of starch and the factors which enter into the preparation of uniform columns It has been possible to define procedures which have given fully reproducible resolving power from column to column and with different preparations of potato starch The columns can be scaled up to 8 cm in diameter without loss of efficiency

The authors wish to acknowledge the assistance of Miss Enid Mellquist and Mr H R Richter in the performance of this work

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PHOTOMETRIC NINHYDRIN METHOD FOR USE IN THE CHROMATOGRAPHY OF AMINO ACIDS

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For the investigations on the chromatographic separation of amino acids outlined in the preceding communication (1), it was necessary to have available a suitable quantitative method for the determination of the concentration of amino acids in the effluent from the column For this purpose, the method should be sufficiently general to include the determination of most of the amino acids and peptides likely to be encountered in protein hydrolysates or other material of biological origin The method should have as high a sensitivity as possible to permit the determination of low concentrations of amino acids in the effluent from the chromatogram Also the laboratory procedure should be fairly simple to permit the method to be applied conveniently to large numbers of effluent samples

It appeared probable that a photometric method would best fulfil these requirements The two colorimetric methods of this type which had received the most study were the procedures based on the use of β-naphthoquinonesulfonic acid and ninhydrin (triketohydrindene hydrate) as reagents For reasons which will be described, the ninhydrin reaction was selected for further investigation

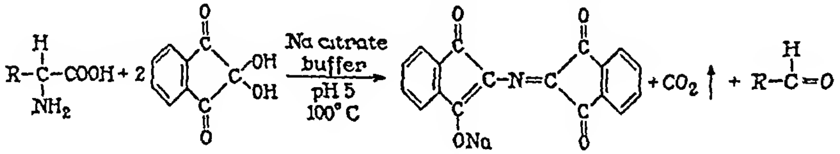
The color reaction between α-NH_2 acids and ninhydrin has been studied extensively in the past It has been established that colored compounds are formed not only with amino acids, but also with peptides, proteins, and other classes of substances possessing free amino groups The reaction is known to be extremely sensitive for qualitative work In earlier attempts to render the color reaction quantitative (2–8), however, it has been found that the color yield per microgram of amino acid decreased markedly as the concentration of amino acid was reduced In addition, the results have not been reproducible In the present investigations, it has been observed that, when the color development is carried out in tubes exposed to the air, these difficulties appear to result primarily from the influence of dissolved oxygen Improved results can be obtained when the reaction is performed in tubes evacuated to 20 mm Under these conditions, the relationship between color yield and amino acid concentration is more nearly linear, although the deviations are still marked By the addition of a strong reducing agent directly to the reaction medium, however, the oxidative side reaction has been eliminated In preliminary experiments

the reduced form of ninhydrin, as hydrindantin (9), was added. Subsequently, it proved simpler to add stannous chloride to the reaction mixture. The stannous chloride serves to reduce part of the ninhydrin, and the preparation of hydrindantin in crystalline form is not required. Although ascorbic acid used as an antioxidant increases the sensitivity of the reaction for qualitative use (10), it is unsuitable for quantitative work because it can also give colored condensation products with amino acids (*cf.* (11)).

When conditions had been established which would give thoroughly reproducible photometric readings with a given amino acid, a study was made with several amino acids to determine the effects upon the reaction of variations in pH, temperature, time of heating, and amounts of reagents. At the same time, the mechanics of the procedure were developed to permit the analysis of large numbers of samples in a routine manner.

The method still possesses one important disadvantage. Although reproducible results are obtained for a given amino acid, the different amino acids do not all yield the same amount of color per mole. For chromatographic analysis, this is not a serious disadvantage. In those instances in which the starch column can separate an amino acid completely from the other components of the mixture, the ninhydrin reaction can be made to give quantitative values by the use of a factor appropriate for the amino acid in question.

Some of the possible causes for variations in the color yield per mole have been studied. The absorption spectra indicate that all the α-NH_2 acids (except cysteine) give the same major colored end-product. This blue coloring matter was prepared by Ruhemann (9) by the reaction of ninhydrin with alanine and by the condensation of hydrindantin with ammonia. To this compound Ruhemann assigned the structure of diketohydrindylidene-diketohydrindamine. In the present work, the product has also been isolated from the mixtures obtained in the reactions of ninhydrin with glycine and glycylleucine. The substance crystallizes as the sodium salt from the



α-NH_2 acid Ninhydrin Diketohydrindylidene-diketohydrindamine Carbon dioxide Aldehyde

citrate buffer solution used in the present experiments. The absorption spectrum of a standard solution of diketohydrindylidene-diketohydrindamine can be compared with the spectrum of the unfractionated reaction mixtures from ninhydrin and α-NH_2 acids. The comparison shows that in the leucine reaction, for example, the colored product is formed in only 93

per cent of the theoretical yield A modification of the method which would render the yields quantitative in all cases would be an improvement For a given amino acid, the percentage yield of the colored product is independent of the initial amino acid concentration This fact indicates that the low yield is characteristic of the mechanism of the reaction under the experimental conditions employed and is not due to destruction of part of the color by a trace of oxygen

With proline and hydroxyproline, as shown by Grassmann and von Arnim (12), the reaction follows a different course than with the amino acids containing an α-NH_2 group These two amino acids give products with a maximum absorption at 440 mμ The present procedure can also be used to determine proline and hydroxyproline, although the sensitivity is less than in the case of the amino acids which form diketohydrindylidene-diketohydrindamine

It has long been known that colorimetric ninhydrin methods are not specific for the NH_2 groups of amino acids The NH_2 groups in peptides give good color development, many amines such as histamine and tyramine will react and the presence of hydrindantin, used in this procedure, causes NH_3 to give a nearly quantitative yield of the blue reaction product For chromatographic experiments with amino acids and peptides, the generality of the reaction extends its usefulness For work with unfractionated biological material, the lack of specificity would be a disadvantage, as was recognized by Harding and MacLean (2) The specificity of the photometric ninhydrin method is similar in a number of respects to that of the nitrous acid reaction for amino nitrogen The method may be of value in instances when the nitrous acid reaction is useful For the estimation of free amino acids in the presence of peptides, the photometric method, of course, lacks the specificity of the gasometric amino acid carboxyl determination of Van Slyke, Dillon, MacFadyen, and Hamilton (13)

The photometric ninhydrin method, with the present modifications which have rendered the results fully reproducible, appears to possess some advantages over β-naphthoquinonesulfonic acid procedures for those applications for which these methods are suitable The reaction of amino acids with β-naphthoquinonesulfonic acid, as employed by Folin, recently modified by Frame, Russell, and Wilhelmi (14), and compared with the CO_2 method by Chinard and Van Slyke (15), involves the additional operation of bleaching of the excess reagent The ninhydrin reagent solution possesses the advantage of being stable, and for routine use can be stored under nitrogen for a month or more Fading of the color in the ninhydrin method proceeds at a much slower rate than that reported for the naphthoquinone procedure (15) The ninhydrin reaction yields the same end-product from all the α-NH_2 acids (cysteine excepted), whereas the chemistry of the β-naphtho-

quinonesulfonic acid reaction is less well defined and the absorption maxima of the colored products obtained from different amino acids, though similar, are not identical (14)

Apparatus

Pipettes—For the pipetting of large numbers of small samples of amino acid solutions for analysis, modified self-adjusting transfer pipettes are used in 0 05, 0 1, 0 2, and 0 5 cc sizes [1] The accuracy of these pipettes is increased by operating them on a manifold connected to both compressed air and vacuum lines For long series of analyses, this arrangement is also much more convenient for the operator The arrangement of the pipetting stand is illustrated in Fig 1 The manifold is made from three T-tubes The third tube is mounted behind the rubber stopper (size 13) The connections are made with soft rubber hemocytometer pipette tubing A slight vacuum (or pressure) is applied to the micro pipette by touching the top of the appropriate T-tube and greater vacuum (or pressure) by simultaneously pressing on the adjacent rubber tubing The stop-cocks are closed only during the adjustment of the vacuum to about 60 mm and the pressure to about 15 mm Before use, the tip of the micro pipette should be bent, as in Fig 1, and fire-polished to give a delivery time of 8 to 12 seconds at 15 mm pressure At this rate, and with wiping of the pipette tip before delivery, the reproducibility of delivery is 0 1 to 0 2 per cent The hold up is about 2 per cent A series of tubes from a chromatogram is run through without washing the pipette between samples For each solvent employed the pipette must be calibrated gravimetrically A table of calibration factors expressed in terms of the fraction of the rated delivery is prepared for each pipette The metal holders for the 150 × 18 mm sample tubes and photometer tubes are cut from brass tubing of 20 mm inner diameter

Photometer Tubes—For the chromatographic procedure, it has been necessary to accumulate a matched set of over 1000 tubes for use with the Coleman junior spectrophotometer, model 6-A The tubes have been selected from strain-tested soft glass test-tubes, 150 × 18 mm, without lips [2] A solution of methyl red in 0 03 N HCl is prepared of such a strength as to give a reading of 0 60 to 0 70 on the optical density scale when read at 525 mμ against a water zero About 100 tubes are filled with 5 to 10 cc of the methyl red solution It is important that all tubes receive the methyl red solution from the same reservoir bottle Pouring the solution

[1] The pipettes are made to the design of Dr P L Kirk by the Microchemical Specialties Company, 1834 University Avenue, Berkeley 3, California (catalogue No 283-B)

[2] Catalogue No 9446, Arthur H Thomas Company, Philadelphia, Pennsylvania

from tube to tube can introduce significant errors The largest group of tubes giving readings within 0 005 unit of the same value is selected The side of the tube facing the light source is marked temporarily at the time of the test, and subsequently with a glass-marking tool,[3] to indicate the correct position for the tube in the spectrophotometer About a dozen of

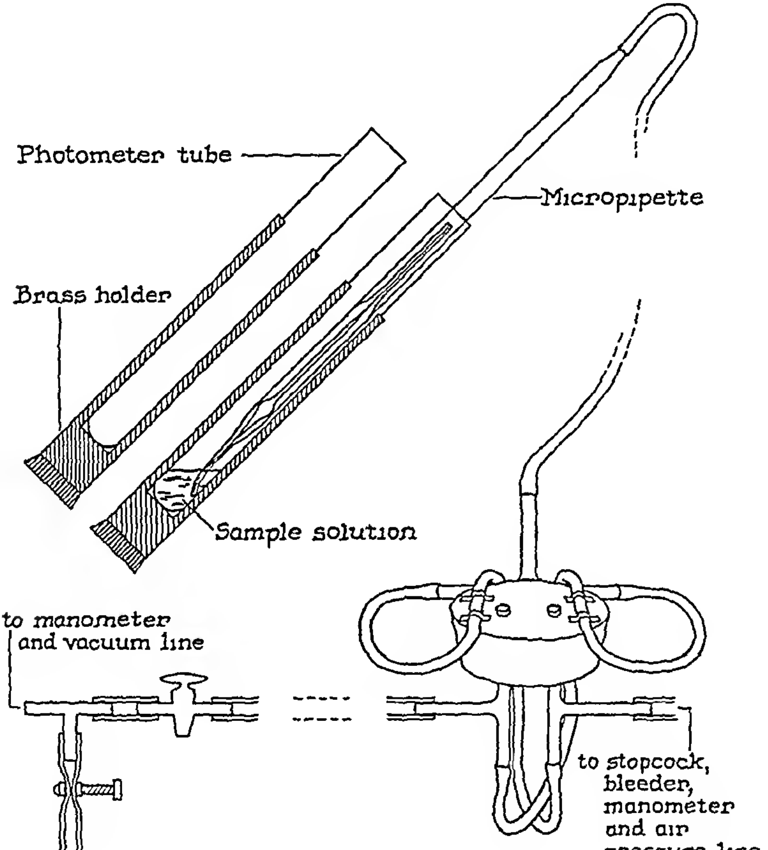


FIG 1 Arrangement for the pipetting of samples for analysis

these tubes are marked as standards and reserved for use only in the checking of new sets

In calibrating subsequent groups of 100 tubes against standard tubes filled with methyl red solution, each new tube is rotated in the instrument to determine whether it can be set to read within 0 005 unit of the standard

[3] Catalogue No 300S, Chicago Wheel and Manufacturing Company, 1101 West Monroe Street, Chicago, Illinois, with additional grinding wheels No 5 B-44

reading The satisfactory tubes are appropriately marked for position The selected tubes are permanently numbered in sets of 200 tubes each Each set can be subjected to an additional test by observing the zero reading when the tubes are filled with distilled water In general, tubes selected by the methyl red procedure have given uniform zero readings

When measured by calipers below the rim, the tubes selected for this study had an inner diameter of 16 25 ± 0 15 mm The outer diameter averaged 18 3 mm Once a calibrated set has been selected with tubes of a given average diameter, such as 18 3 mm , it is necessary that subsequent lots of tubes obtained from the manufacturer have an average diameter in the same range Under these conditions, twenty-five to 50 tubes per hundred may prove acceptable If such specification is not made, some lots may run all high or all low

When the sets have been handled in wooden or aluminum racks,[4] in order to protect the tubes from being scratched, and washed with boiling soap solution, the tubes have remained accurate during constant use for more than a year

To cover the photometer tubes during the analysis, Aloe-Willett aluminum caps[5] are used

Water Bath—A vigorously boiling water bath is required An open bath can be used, but the amount of evaporation of the solvent (*cf* Table III) from the tubes is less reproducible The present experiments have been carried out in a specially designed, electrically heated, covered bath[6] with a constant level regulator For these analyses, the bath is operated with the thermostat at the maximum setting, so that heat is constantly, not intermittently, applied The tubes are immersed to a depth of about 2 inches The rate of heat supply should be sufficient to bring the bath back up to 99–100° within 2 minutes after the insertion of a full rack of 50 tubes Only one rack should be inserted at a time The rear corners of the cover are bent downward slightly to allow the escape of steam without the collection of condensed water A double hook handle for insertion and removal of the racks can be made from a $\frac{1}{4}$ inch metal rod

Pipetting Machines for Reagents—For a small number of analyses, the reagents can be added by a burette or pipette When large numbers of samples are being run, the ninhydrin solution can be stored under nitrogen in a 1 liter brown or red glass reservoir attached to a pipetting machine [7]

[4] Suitable aluminum racks are described in the preceding communication (1)

[5] Catalogue No JL-78300 (for 18 mm tubes) A S Aloe Company, St Louis, Missouri

[6] Catalogue No 405/3/R, Electric Heat Control Apparatus Company, 507 Fifth Avenue, New York 17

[7] Model No 40 SS-10, Brewer Automatic Pipetting Machine, Baltimore Biological Laboratory, Inc , 500 North Calvert Street, Baltimore 2, Maryland

The flexible connections are made with $\frac{1}{8}$ inch inner diameter Neoprene tubing A 250 cc dropping funnel, for use in filling the reservoir, is mounted on the bottle through a 3-hole rubber stopper The reservoir is connected to the top of a second bottle of the same size by a U-tube, and the second bottle is connected through a 2-hole stopper to a third 1 liter bottle by a U-tube which reaches to the bottom of each The second bottle is initially filled with water, and the air in the system is replaced by nitrogen introduced through the dropping funnel at the time the apparatus is set up The reservoir can be refilled without replenishing the nitrogen, except when the apparatus is disassembled for cleaning The pipetting machine is equipped with a 3 cc syringe and set for 1 cc delivery The valves (ungrooved) may require regrinding by hand with a fine emery or rouge to insure smooth performance with organic solvents Fire polishing of the glass delivery tip, when small volumes such as 1 cc are being delivered, helps to eliminate a hanging drop or back flow

A second pipetting machine, equipped with a 10 cc syringe, is used to deliver 5 cc of the diluent solution from a 2 liter storage bottle The flexible connections can be made with 3/32 inch inner diameter Tygon tubing or $\frac{1}{8}$ inch Neoprene tubing For convenience in the analysis of large numbers of samples, the reaction mixtures are diluted with a given volume of solvent from the pipetting machine to avoid the procedure of bringing the solutions to a prescribed volume in calibrated glassware

Reagents

Ninhydrin—To insure a low blank reading in the photometric procedure, the ninhydrin, prepared commercially according to the method of Teeters and Shriner (16), has usually been recrystallized within several months of the time of use To 250 cc of water, 100 gm of ninhydrin are added The hot solution is treated with about 5 gm of decolorizing carbon The filtrate is stored at 4° overnight The ninhydrin is washed on the filter four or five times with 20 cc portions of cold water The air-dried crystals are stored in dark glass The recovery is 85 to 90 per cent

Citrate Buffer—The buffer, pH 5 (0 2 M), is prepared from 21 008 gm of citric acid, $C_6H_8O_7 \cdot H_2O$ (reagent grade) and 200 cc of N NaOH diluted to 500 cc Several times this quantity can be prepared and stored in the cold with thymol The pH of the buffer when diluted with an equal volume of water should be 5 0 ± 0 1

Methyl Cellosolve—The samples of methyl cellosolve should give a clear solution when mixed with an equal volume of water[3] and should give a negative or very faint peroxide test with 10 per cent aqueous KI

[3] Turbidity from several samples of methyl cellosolve was found to result from the fact that the solvent had been repackaged in lacquered cans Purchase of the solvent packaged in glass or in the manufacturer's original containers is to be preferred

Ninhydrin Solution—Dissolve 0.80 gm. of reagent $SnCl_2 \cdot 2H_2O$ in 500 cc. of the citrate buffer, pH 5. Add this solution to 20 gm. of recrystallized ninhydrin dissolved in 500 cc. of methyl cellosolve. Transfer the reagent solution to the reservoir bottle. If the system is not already filled with nitrogen, run a stream of nitrogen through the dropping funnel for about 30 minutes. When stored in this manner the solution can be kept for at least a month without deterioration. The adequacy of the preservation of hydrindantin in the reagent solution can be checked by determining the color yield on a standard amino acid solution of relatively low concentration (1 mM). If unrecrystallized ninhydrin is used, and the reagent solution is clarified with carbon, high blanks are likely to result from nitrogenous materials on the carbon.

If only a few analyses are being run at one time, and a pipetting machine is not required, the necessary small quantity of the reagent solution can be prepared for immediate use and the 1 or 2 cc. aliquots run into the photometer tubes from a burette.

Diluent Solution—Mix equal volumes of water and *n*-propanol (C.P.).

Standard Amino Acid Solutions—For the analysis of 0.1 cc. samples, 1.6 to 2.0 mM solutions of amino acids are convenient. The solutions are diluted 1:1 or 1:4 for analyses in which 0.2 or 0.5 cc. samples are required. When water-alcohol solvents are employed, the amino acids are dissolved in water and the solutions made to the appropriate volume with the dry organic solvent. A small amount of HCl is added to dissolve tyrosine and cystine. Blank solutions for each solvent mixture are prepared at the same time.

Procedure

In the case of standard solutions of amino acids, triplicate samples of the blank and the standards are pipetted into a series of photometer tubes. When the effluent from a chromatogram is being analyzed, a single sample is pipetted from each fraction. If an automatic fraction collector (1) has delivered the appropriate size of sample directly into the photometer tubes, the pipetting step is eliminated.

If the solutions for analysis are acidic, they should be neutralized (methyl red) to within ±0.1 cc. of 0.1 N NaOH. For a series of tubes from a chromatogram, a preliminary titration is made on a blank sample. Subsequently, 1.0 N NaOH is appropriately diluted so that 1 or 2 drops (0.05 to 0.1 cc.) from a burette will bring the sample within the desired limits. The prescribed number of drops of the alkali of adjusted concentration is added to each tube of the series prior to analysis. Acidic solutions will usually pick up a trace of NH_3 from the air. Since as little as 0.1 γ of NH_3 can be detected by the present procedure, it is important that the blanks and the analytical samples be handled under strictly parallel conditions.

The pipetting machine for the ninhydrin reagent is checked for delivery by running five aliquots into a 5 cc volumetric flask This is done daily before use of the machine, and also serves to discard the solution which has been standing in the Neoprene tubing The delivery should be within ±0 5 per cent of the set value One or two aliquots of the ninhydrin solution (1 cc for 0 1 and 0 2 cc samples, 2 cc for 0 5 cc samples) are run into the photometer tubes With samples in water or in solvents miscible with water, 1 cc of ninhydrin solution can be used for 0 5 cc samples if accuracy to the last few per cent is not important Aluminum caps are placed on the tubes and the tubes are shaken to mix the reagent and sample If the shaking is done by machine, the rack can be placed for 30 seconds on a reciprocal shaker[9] operating at about 240 excursions per minute

The rack of tubes is heated for 20 minutes in a vigorously boiling water bath The pipetting machine for the water-propanol diluent is checked for delivery with a 25 cc volumetric flask 5 cc (±0 03 cc) of the diluent are added to each tube The stream is directed into the center of the solution to give good mixing The tubes are wiped and transferred to a dry rack The tubes are shaken by hand or by machine (1 minute) Readings are taken on the spectrophotometer starting at about 15 minutes after removal from the bath Readings are essentially constant for 1 hour after completion of the reaction The analyses should be run in groups of not more than 50 tubes to permit the readings to be completed within this time

The tubes are read on the Coleman junior spectrophotometer, model 6-A, at 570 mμ The blanks are read against a reference tube of 1 1 propanol-water The average blank tube is chosen, and the rest of the series read with the instrument set on the blank determination as zero The blank reading should be about 0 08 to 0 10 on the optical density scale for 1 cc of reagent and 0 15 to 0 20 for 2 cc of reagent Proline and hydroxyproline are read at 440 mμ

For tubes which read near to or above 1 00, the solutions and the blank are diluted with additional 5 cc samples of the propanol-water solution When the volume in the tube reaches 16 to 17 cc , shaking by machine is not satisfactory, and inversion of the Neoprene-stoppered tubes is required If the readings are still off the scale (above 1 00), samples are pipetted into other photometer tubes for further dilution

When an analysis is completed, the tubes are rinsed with water, a monel wire screen or a perforated aluminum cover being used to permit the draining of a full rack of 50 tubes in one operation The tubes are half filled with 0 2 per cent aqueous solution of soap flakes The rack of tubes is heated for 20 minutes in a boiling water bath The tubes are rinsed three times with water and dried in an oven at 110° The boiling soap solution is

[9] Catalogue No 5855, Precision Scientific Company, Chicago, Illinois

required to remove the band of material that is deposited on the walls of the tubes when volatile solvents are used.

Calculations

A standard curve is plotted for 0.1 cc. aqueous samples of leucine at six concentrations varying from 0.5 to 2.0 mM. Before being plotted, the average values are divided by the pipette calibration factor for water to give corrected readings for 0.100 cc. samples. From the graph, a table is prepared giving the millimolar concentrations corresponding to optical density readings from 0.01 to 1.00, in steps of 0.01 unit. The concentrations are multiplied by 11.1/6.1 and 16.1/6.1 to give concentrations corresponding to the readings obtained after dilution of the 6.1 cc. with one or two additional 5 cc. aliquots of the diluent. A condensed format of the standard table obtained with tubes possessing an inner diameter of 16.25 mm. is given in Table I, which covers concentrations up to 5.4 mM. The procedure can be extended by manual dilutions to much higher concentrations. The curve follows Beer's law through readings up to an optical density of about 0.50. There is a deviation of 4 per cent from the straight line relationship at an optical density of 1.0.

TABLE I

Relationship of Optical Density to Leucine Concentration (Condensed Table of Leucine Equivalents)

Determined on 0.100 cc. aqueous samples in photometer tubes of 16.25 mm. inner diameter.

Spectrophotometer reading, optical density × 100	Leucine concentration, mM per liter			Spectrophotometer reading, optical density × 100	Leucine concentration, mM per liter		
	Volume of diluent added				Volume of diluent added		
	5 cc.	10 cc.	15 cc.		5 cc.	10 cc.	15 cc.
10	0.196	0.357	0.518	60	1.18	2.15	3.11
20	0.392	0.714	1.03	70	1.39	2.53	3.67
30	0.588	1.07	1.55	80	1.61	2.93	4.25
40	0.784	1.43	2.07	90	1.83	3.33	4.83
50	0.980	1.78	2.59	100	2.05	3.73	5.42

For the other amino acids and related compounds, the color yields per mole can be expressed relative to the leucine value as 1.00. For 0.1 cc. aqueous samples, the millimolar concentrations read from Table I, corrected for pipette delivery, were divided by the millimolar concentrations of the standard solutions to give the yields summarized in Table II. Each amino acid and peptide listed in Table II was checked for correct elementary analysis and, whenever possible, for optical rotation (*cf.* (1)). The other sub-

stances were obtained from commercial sources and were not purified before analysis

For other than 0 1 cc aqueous samples, the values given in Table I for millimolar concentration corresponding to a given spectrophotometer reading require correction for the changes in volume involved Before

TABLE II

Color Yields from Amino Acids and Other Compounds on Molar Basis Relative to Leucine

Determined on 0 1 cc aqueous samples of 2 0 mM solutions, heating time, 20 minutes, read at 570 mμ

Compound	Color yield	Compound	Color yield
Alanine	1 01	Glutathione	0 76
Arginine	1 00	Glycine ethyl ester	1 00
Aspartic acid	0 88	Glycyltyrosine	0 88
Citrulline	1 03	Glycylphenylalanine	1 04
Glutamic acid	1 05	Glycylglycine	0 89
Glycine	1 01	Glycylleucine	1 05
Histidine	1 04	Leucylglycine	0 92
Isoleucine	1 00	Phenylalanylglycine	0 97
Leucine	1 00	Phenylalanine ethyl ester	0 98
Lysine	1 12	Histamine	0 65
Methionine	1 00	Taurine	0 97
Phenylalanine	0 88	Tyramine	0 64
Serine	0 94	Sarcosine	0 84 *Ca*
Threonine	0 92	Glucosamine	1 00
Tyrosine	0 88	Creatine	0 03
Valine	1 02	Creatinine	0 03
Cysteine	0 15 *Ca*	Dibenzylamine	0 04
Half cystine	0 54	Glycine anhydride	0 01
Tryptophan	0 72 *Ca*	Urea	0 03
Proline	0 05	Adenine	0 00
Hydroxyproline	0 03	*p* Aminobenzoic acid	0 00
Ammonia	0 98 *Ca*	Diethylbarbituric acid	0 00
Asparagine	0 94	Glucose	0 00
Glutamine	0 99	Uric acid	0 00

calculating the correction factors to be applied to the analysis of samples containing volatile organic solvents, it is necessary to determine gravimetrically the amount of evaporation during the analysis by weighing tubes before and after heating the reaction mixture for 20 minutes under the experimental conditions employed in an actual determination With butanol-water and propanol-water samples, essentially the entire 0 1 to 0 5 cc sample of solvent evaporates during the heating process If acidic

samples are neutralized before analysis, the volume of NaOH solution added must be included in the calculation. When each term is expressed in cc., the correction factors (F) are calculated as follows

$$F = \frac{\begin{array}{c}\text{(Sample volume + neutralizing solution} \\ \text{+ ninhydrin solution + diluent)} - \text{(loss by evaporation)}\end{array}}{1.1 + \text{diluent}} \times \frac{0.100}{\text{sample volume}}$$

Representative factors for two organic solvents are given in Table III. As a first approximation, these factors, used in conjunction with the rela-

TABLE III

Factors for Different Sample Sizes and Solvent Systems

Factors by which the millimolar concentrations from Table I are to be multiplied to give corrected leucine equivalents

Solvent	Sample size	Ninhydrin solution	Loss by evaporation		Factor†		
					Volume of diluent added		
					5 cc	10 cc	15 cc.
	cc	*cc*	*mg*	*cc* *			
Water	0.1	1	(14)‡		1.000	1.000	1.000
	0.2	1	(19)		0.508	0.504	0.502
	0.5	2	(19)		0.246	0.225	0.217
Butanol-water§	0.1	1	94	0.10	0.984	0.991	0.993
	0.2	1	194	0.20	0.492	0.495	0.496
	0.5	2	395	0.45	0.231	0.217	0.212
Butanol-benzyl alcohol-water ‖	0.1	1	41	0.03	0.996	0.998	0.998
	0.2	1	62	0.05	0.504	0.503	0.502
	0.5	2	79	0.07	0.244	0.224	0.216

* Approximate.

† To be divided by the calibration factor of the pipette.

‡ The small loss from water samples is subtracted from the loss with organic solvent samples in the calculation of approximate volume change.

§ Butanol-water containing 150 cc. of water per liter ($d^{25} = 0.838$).

‖ Butanol-benzyl alcohol-water 1:1:0.288 by volume ($d^{25} = 0.936$).

tive yields of color listed in Table II, give satisfactory results for most of the amino acids in these solvents, if an accuracy greater than ±5 per cent is not required. The color yields given in Table II and the factors listed in Table III may also be used for approximate results with other solvents that may be tried with starch chromatograms. Under such circumstances, the factors given in Table III for water or the butanol-benzyl alcohol solvent may be used for relatively non-volatile solvents, and the butanol factors for volatile solvents.

However, for accurate quantitative work with those solvents which are

selected as optimum for chromatographic analyses, the exact degree of evaporation must be determined experimentally, and the color yields obtained from a given amino acid must be checked by the user with standard solutions made up in the organic solvent As may be seen from Table IV, the yields thus obtained may differ by a few per cent from the values obtained with aqueous samples given in Table II The yields should be determined on the same size of sample being used in the chromatographic experiments, although no differences have been observed between 0 1 cc and 0 5 cc samples in the solvents studied thus far For accurate work with solvents other than water, therefore, the concentration of amino acid given in Table I is multiplied by the appropriate factor from Table III and divided by the appropriate color yield from Table IV

TABLE IV

Color Yields from Amino Acids in Organic Solvent Solutions on Molar Basis Relative to Leucine in Water

Determined on 0 1 cc to 0 5 cc samples, heating time, 20 minutes

Amino acid	Color yield	
	Butanol water solvent	Butanol benzyl alcohol water solvent
Leucine	0 99	1 01
Isoleucine	1 00	1 03
Phenylalanine	0 85	0 86
Tyrosine	0 86	0 87
Methionine	1 00	1 01
Valine	1 01	1 03

The factors given in Table III and the color yields listed in Table IV are fully reproducible when once determined under given experimental conditions Except with NH_3 and tryptophan, it is not necessary to run controls with each batch of analyses If this were not the case, the application to chromatography would be rendered unduly complicated by a need for repeated controls An occasional check on the recovery of leucine from a known solution serves to confirm the reproducibility of the procedure

The calculation of the recovery of leucine from a synthetic mixture which has been subjected to chromatographic analysis is given in Table V Suitable data sheets are mimeographed to facilitate the handling of the results In this example, the automatic fraction collector has been used with a column 0 9 cm in diameter to deliver approximately 0 5 cc samples directly to photometer tubes

When aliquots are pipetted for analysis from larger effluent fractions, the summations of the uncorrected millimolar concentrations are multi-

TABLE V

Data Sheet, Determination of Leucine

Solvent, 1 1 0 288 butanol-benzyl alcohol-water, ninhydrin solution, 2 cc, fraction collector, 25 drops = 0 504 cc, entire fraction analyzed, wave-length, 570 mμ, readings recorded as optical density × 100

Fraction No	Volume of diluent, 5 cc		Volume of diluent, 10 cc	Volume of diluent, 15 cc	Uncorrected amino acid concentration†
	Read against 1 1 water propanol	Read against blank fraction*			
					mM
11	22 0	0	0	0	
13	22 2	0			
15	21 1	−1			
35	22 0	0			
36	22 0	0			0 00
37	23 5	1 5			0 03
38	29 0	7 0			0 14
39		24			0 47
40		57			1 12
41		100	62		2 22
42		140	90		3 33
43		150	102	81	4 30
44		130	84		3 08
45		77			1 54
46		28			0 55
47	28 5	6 5			0 13
48	22 0	0			0 00
49	22 5	0 5			
50	22 0	0			

Integration Fraction 43 $4\ 30 \times 0\ 216 \times 0\ 5‡ = 0\ 464$

Sum of Fractions 41, 42, and 44 $8\ 63 \times 0\ 224 \times 0\ 5 = 0\ 967$

" " " 37-42, and 46-47 $3\ 98 \times 0\ 244 \times 0\ 5 = 0\ 486$

Total = 1 917 micromoles

Correction for color yield from leucine in this solvent, 1 917/1 01 = 1 898 micromoles = 0 249 mg of leucine

Leucine added, standard aqueous solution of a mixture of amino acids 40 mM with respect to leucine, 0 495 cc (0 5 cc pipette, delivery 99 per cent) diluted to 5 00 cc and 0 485 cc (0 5 cc pipette, delivery 97 per cent) placed on the column in butanol-benzyl alcohol Theoretical yield = 1 920 micromoles = 0 252 mg, recovery = 98 8 per cent

* Fraction 11

† From the expanded form of Table I

‡ If 0 5 cc samples are pipetted, the factor becomes 0 216 × (volume of effluent fraction)/(pipette calibration factor)

plied by the appropriate factors from Table III and by the exact volume of an effluent fraction to give micromoles of amino acid When the whole

fraction is analyzed, as in this example, the sample volume is equal to the volume of an effluent fraction, and the two terms cancel out At large effluent volumes, when the peaks cover twenty or more fractions, integrations can be obtained from the analysis of every second fraction

In order to obtain a graphic picture of the fractionation, the uncorrected millimolar concentrations are used directly for preliminary plotting of effluent concentration curves The graph gives information on the symmetry of the curves and the degree of fractionation The curves are plotted before decision is made on the division of the peaks for integration For publication, the curves in the preceding paper (1) have been replotted, each point being corrected to "leucine equivalents" by means of Tables I and III When the peaks are completely separated, the curves could also be corrected for color yield, but in the case of incomplete separation of the components, this is not possible In the preceding paper (1) the method is given for the calculation of the amounts of each component in a series of partially overlapping peaks

Accuracy—When the calibrations have been carefully made both for the pipettes and the photometer tubes, the readings on 0 2 micromole of an α-NH_2 acid can invariably be reproduced to within 0 02 optical density unit, corresponding to an accuracy of about 2 per cent In the chromatographic analyses, it is necessary to work, in part, below this optimum concentration range In a large series of chromatograms, integration of the effluent curves has given recoveries of 100 $\pm$ 3 per cent, under favorable conditions, and 100 $\pm$ 5 per cent for peaks markedly below the optimum average concentration (1)

Reaction with Proline and Hydroxyproline—The preceding method can be used for the determination of proline and hydroxyproline by measuring the yellowish red products of the reaction at their absorption maximum of 440 mμ However, the optical density readings are only one-fourth and one-seventh, respectively, of those obtained with equimolar solutions of the α-NH_2 acids The color development is only 80 to 90 per cent complete in 20 minutes at 100° Standard curves can be prepared for proline and hydroxyproline with a 30 or 40 minute heating period In the chromatographic analysis, the tubes are usually heated for only 20 minutes along with the rest of the effluent samples As a first approximation, millimolar concentrations can be calculated from Tables I and III, just as in the case of readings at 570 mμ, and the values can be converted to proline by multiplying by the factor 3 7 and to hydroxyproline by multiplying by the factor 7 2 The measurement of proline by the ninhydrin reaction is of course possible only when this amino acid is completely separated from the other amino acids, as it frequently is on the starch chromatograms On the other hand, in view of their low absorption at 570 mμ, small amounts

of proline and hydroxyproline can be present simultaneously with α-NH_2 acids without giving significant interference

Reaction with NH_3—In the oxidative deamination of amino acids by ninhydrin, 1 equivalent of the reagent is reduced in the course of the formation of diketohydrindylidene-diketohydrindamine (9) If oxygen could be completely eliminated by evacuation of the system, the maximum color yields would be obtained from amino acids without the addition of any further source of reduced ninhydrin The yield from NH_3 under these conditions would be low, since, of itself, NH_3 does not give rise to the reduced ninhydrin which is essential for the formation of the colored complex This accounts for the fact that NH_3 does not react positively in a number of the colorimetric ninhydrin procedures that have been used (4, 5, 7) When hydrindantin exists preformed in the reaction mixture, however, as it does in the present procedure, the color yield from NH_3 is in the same range as that from the amino acids In contrast to the amino acid reaction, which is independent of the concentration of hydrindantin above a certain minimum level, the color yield from NH_3 increases with the hydrindantin concentration With the reagent solution used in the present procedure, the color yield from NH_3 reaches about 90 per cent of its maximum value Since different batches of reagent solution may vary somewhat in hydrindantin content, a control determination on a known NH_4Cl solution must be run simultaneously if it is desired to obtain accurate values on NH_3 solutions by the photometric ninhydrin method

Experiments on Color Development

Isolation of Diketohydrindylidene-Diketohydrindamine—The product of the reaction of ninhydrin with glycine at pH 5 was prepared in order to compare its absorption spectrum with that of the unfractionated reaction mixture obtained in the ninhydrin analysis

The amino acid (75 mg), dissolved in 10 cc of water, was heated for 20 minutes at 100° with 700 mg of ninhydrin dissolved in 20 cc of citrate buffer, pH 5 (0 2 M) The product (245 mg) which crystallized from the cooled solution corresponded to 75 per cent of the theoretical yield of the sodium salt of diketohydrindylidene-diketohydrindamine The same procedure carried out with the peptide leucylglycine (188 mg) gave the same product in 40 per cent yield For analysis the sodium salt (50 mg) was recrystallized from about 15 cc of hot 1 1 water-*n*-propanol

$C_{18}H_8O_4NNa$ (325 2)	Calculated	C 66 5, H 2 5, N 4 3, Na 7 08
Prepared from glycine	Found	" 66 2, " 2 6, " 4 4, " 7 07
" " leucylglycine	"	" 66 6, " 2 6, " 4 3, " 6 94

Absorption Spectra—The absorption curves have been determined with 0 1 cc samples of 2 mM aqueous amino acid solutions The final vol-

ume of the reaction mixture was 6 10 cc in photometer tubes of 16 25 mm inner diameter In Fig 2 the curves for leucine, serine, and ammonia are compared with the absorption spectrum obtained from an equimolar solution of the crystalline sodium salt of diketohydrindylidene-diketohydrindamine The solution was prepared by dissolving 1 065 mg of the sodium salt in 100 cc of a mixture of the ninhydrin solution and the propanol-water diluent in the proportions of 1 10 and was read against this solvent as the blank The absorption spectra support the conclusion that the α-NH_2 acids and ammonia yield diketohydrindylidene-diketohydrindamine

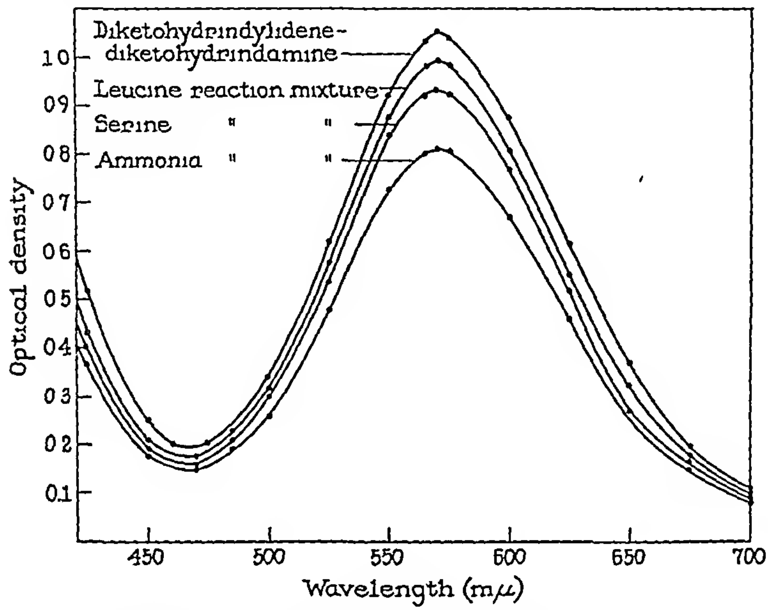


FIG 2 Comparison of the absorption spectrum of diketohydrindylidene diketo hydrindamine with the spectra obtained after the reaction of ninhydrin with equimolar amounts of leucine, serine, and ammonia

under the conditions of the determination The curves obtained with the other α-NH_2 acids, except cysteine, and with peptides, are similar to those shown in Fig 2 The major end-product is the same in all cases, exhibiting an absorption maximum at 570 mμ The differences in the color intensities obtained with the individual amino acids arise from variations in the yield of this product Relative to leucine, the reading of the pure sodium salt corresponds to a color yield of 1 07 (Table II) On this basis, leucine yields about 93 per cent of the theoretical amount of this product The yields for phenylalanine and glutamic acid, for example, are 82 and 98 per cent

Color development with ninhydrin is not specific for amino acids, since a variety of primary amines and some secondary amines will give significant amounts of color. The structure of the end-products in these cases remains to be determined. The absorption curves obtained with histamine and with the N-methyl-substituted amino acid, sarcosine, are given in

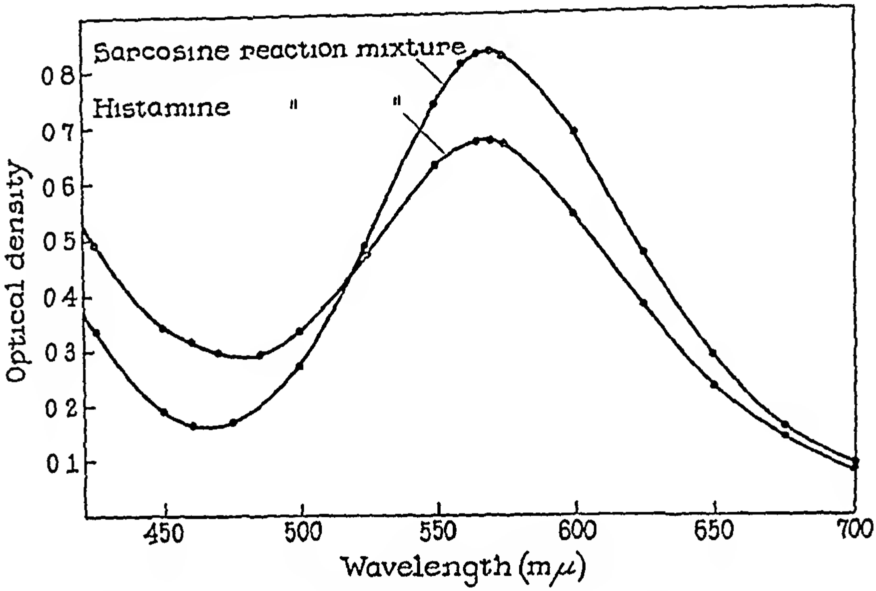


FIG 3 Absorption spectra obtained after the reaction of ninhydrin with sarcosine and histamine

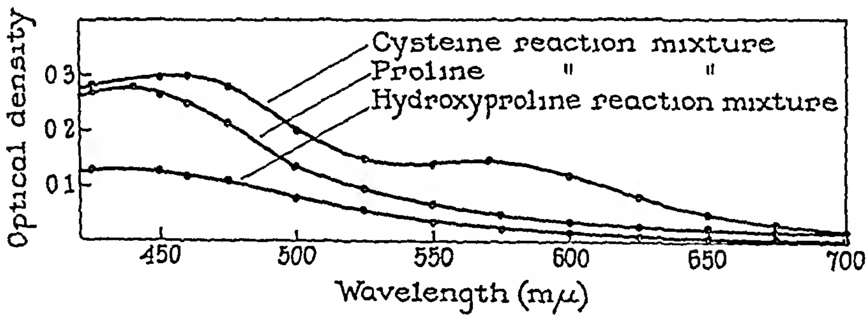


FIG 4 Absorption spectra obtained after the reaction of ninhydrin with proline, hydroxyproline, and cysteine

Fig 3. Both compounds yield products with absorption maxima at 570 mμ.

Cysteine, which has been mentioned as an exception to the general reaction of the α-NH_2 acids, gives an absorption curve which is somewhat similar to that obtained with proline and hydroxyproline (Fig 4). Neutral cysteine solutions, after standing for 24 to 48 hours, give the same color

yield as cystine, with maximum absorption at 570 mμ It is possible that the small amount of absorption in the range of 570 mμ observed with fresh solutions of cysteine may be attributed to the presence of some cystine in the reaction mixture The compounds yielded by the prolines under these conditions presumably correspond to the structures assigned by Grassmann and von Arnim (12)

Variation of pH—The variation of color yield with the pH of the aqueous citrate buffer is shown in Fig 5 The absorption maximum for the α-NH_2 acids is at 570 mμ over the pH range studied The maximum color yield from leucine is obtained at pH 5, which has been chosen for the general procedure

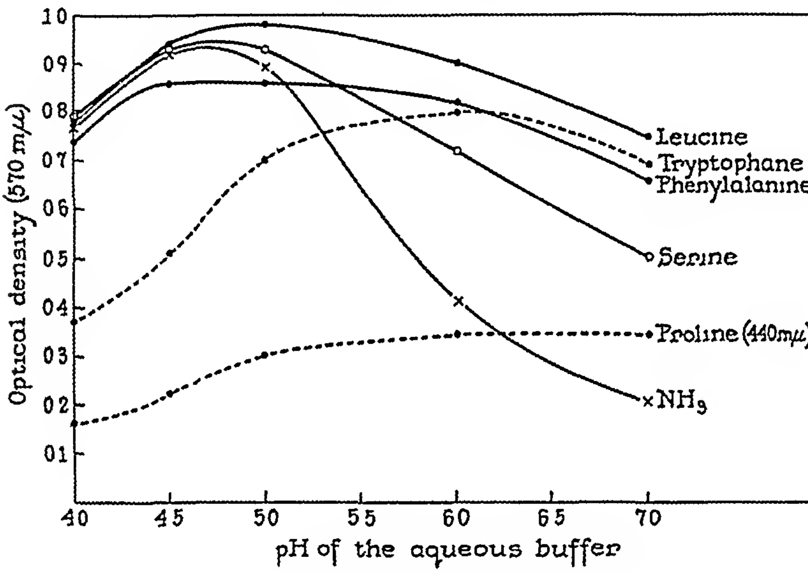


FIG 5 Effect of pH on the intensity of color obtained after the reaction of ninhydrin with amino acids and ammonia

The pH optima for the other α-NH_2 acids fall close to this value, with the exception of tryptophan which gives a maximum yield at pH 6 For most of the amino acids, a change in pH of 0 1 unit at pH 5 0 introduces less than a 1 per cent deviation in the optical density reading

Rate of Reaction—The rates of color development have been determined for representative α-NH_2 acids over a range of pH from 4 to 7 In all cases, at 100° the reaction was complete in less than 20 minutes The color yields were unaltered by an increase in the ninhydrin concentration This result parallels the observations on the heating periods required for the complete liberation of CO_2 from amino acids at a ninhydrin concentration of 20 mg per cc in the gasometric method of Van Slyke, Dillon, Mac-

Fadyen, and Hamilton (13) A more detailed study of the rate of color development has been made at pH 5 Constant readings are obtained with leucine in 5 minutes and with alanine in 10 minutes The reaction with glycylphenylalanine and glycylleucine is complete in 20 minutes On the other hand, phenylalanylglycine and leucylglycine reach only 90 per cent of completion in this time Primary amines, such as ethylamine and ethanolamine, react still more slowly The time of 20 minutes has been chosen as a heating period which gives constant readings with all of the α-NH_2 acids and can be expected to give reasonably high readings with most peptides possessing a free NH_2 group

Temperature—The color yields are lower if the reaction is carried out at temperatures below 100° For leucine, isoleucine, tyrosine, and phenylalanine, the optical densities were 4 per cent lower when the analysis was carried to completion in a water bath maintained at 95°

Stability of Color—The rate of fading of the blue color is illustrated by the following average readings obtained on leucine samples at the specified times after removal of the photometer tubes from the heating bath 15 minutes 0 835, 30 minutes 0 830, 45 minutes 0 835, 60 minutes 0 835, $1\frac{1}{2}$ hours 0 815, $2\frac{1}{2}$ hours 0 810, 4 hours 0 795, $5\frac{1}{2}$ hours 0 785, and 22 hours 0 710 Thus, the color is stable for about 60 minutes, after which time there is a gradual fading, averaging approximately 1 per cent per hour The end-product is not highly sensitive to oxidation by air, whereas, as noted below, an intermediate in the reaction appears to possess much greater sensitivity

In early experiments, water was used as the diluent and marked fading was noted as a result of precipitation of the sodium salt of diketohydrindylidene-diketohydrindamine The use of 1 1 water-*n*-propanol as the diluent serves to keep the relatively insoluble reaction product in solution

Effect of Stannous Chloride—When 2 mM leucine samples are analyzed with a ninhydrin solution from which the stannous chloride has been omitted, the color yield is about half that obtained in its presence At lower leucine concentrations the percentage decrease in yield is greater By carrying out the reaction in vessels evacuated to 20 to 30 mm, as is done in the gasometric ninhydrin method (13), the color yield from 2 0 mM leucine solutions can be raised almost to the maximum value At a leucine concentration of 0 05 mM, however, the results still run about 10 per cent low

The first trials on the blocking of the oxidative side reaction by the addition of a reducing agent to the ninhydrin solution were made with hydrindantin Consistent results were obtained at a hydrindantin concentration of 1 mg per cc The color yields were unaltered by a 4-fold increase in this concentration Since hydrindantin can be prepared by the action

of stannous chloride on ninhydrin, the addition of stannous chloride directly to the reagent solution was tried and found to give the same results However, the presence of hydrindantin, which is highly insoluble in water, required the addition of an organic solvent which would keep this compound in solution during the course of the reaction and during storage of the reagent solution Among the solvents tested, methyl cellosolve had the highest solvent power for hydrindantin The solvent mixture chosen (1 1 water-methyl cellosolve) does not evaporate in the water bath at 100° and does not precipitate sodium citrate from the buffer

SUMMARY

The reaction of ninhydrin with NH_2 groups to give diketohydrindylidene-diketohydrindamine has been utilized as the basis for a photometric determination of amino acids and related compounds in effluent samples from starch chromatograms The color yields have been rendered fully reproducible by the incorporation of hydrindantin or stannous chloride in the reagent solution to eliminate oxidative side reactions Although the color yield from a given amino acid is constant, the different amino acids do not all give the same percentage yield of the blue product This fact does not prevent the accurate use of the method in chromatographic work in those cases in which the individual amino acids are separated from one another by the fractionation process

Color development is obtained with a variety of compounds containing NH_2 groups, including amino acids, peptides, primary amines, and ammonia For chromatographic work, the generality of the reaction extends its usefulness For work with unfractionated biological material, the lack of specificity is a disadvantage

The reaction is carried out at pH 5 and 100° The absorption maximum of the blue product is at 570 mμ On individual amino acids the accuracy is 2 per cent for samples in the range of 2 5 γ of α-NH_2 nitrogen The mechanics of the procedure have been developed to permit the analysis of a large number of samples on a routine basis

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OXIDATION OF GLUCOSE LABELED WITH RADIOACTIVE CARBON BY NORMAL AND ALLOXAN-DIABETIC RATS*

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The nature of the metabolic defect in diabetes has been vigorously debated for well over 30 years, but despite the interesting new lights shed on it during the past few years (1, 2) *rigid* proof as to whether the derangement in this disease results from an impaired capacity of the diabetic animal to convert glucose to CO_2 is still lacking With the introduction of isotopic carbon, it became possible for the first time to study the direct conversion of carbon-containing compounds to CO_2 We wish to report here observations dealing with the fate of the carbon of administered radioglucose in normal and alloxan-diabetic rats The evidence obtained indicates that the over-all rate of oxidation of glucose by the alloxan-diabetic rat need not differ significantly from that in the normal rat

EXPERIMENTAL

Production and Care of Diabetic Rats—The first five rats (Table I) were injected intraperitoneally with a 2 per cent aqueous solution of alloxan A single injection of 200 mg per kilo of body weight was found effective in producing glycosuria and at the same time kept mortality at a minimum However, the intraperitoneal route of administration produced adhesions in the abdominal cavity, and for this reason the alloxan was injected intravenously in the other animals A dose of 50 mg of alloxan per kilo of body weight, administered as a 5 per cent solution in isotonic saline, was used for intravenous purposes Higher dosages resulted in undue mortality, whereas lower doses frequently failed to induce diabetes

The animals were kept in individual cages in a warm room The stock diet (which consisted of 68 5 parts of wheat, 5 of casein, 10 of fish meal, 1 5 of salt, 5 of a fish oil, and 10 of alfalfa) was supplemented twice weekly with lettuce

Urine was collected daily and preserved with toluene Glucose in urine was determined on the day of collection by oxidation with potassium ferri-

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cyanide and subsequent titration with ceric sulfate (3, 4) Blood glucose was determined by the same method on an aliquot of a protein-free filtrate prepared with $ZnSO_4$ and NaOH (5) The last traces of zinc were removed by the addition of sodium carbonate [1]

The duration of diabetes, degree of glycosuria, and weight changes that occurred in the nine diabetic rats used in this study are recorded in Table I

Preparation of C^{14}-Labeled Glucose[2]—C^{14}-labeled starch and glucose were isolated from tobacco leaves which had been illuminated in the presence of $C^{14}O_2$ Labeled glucose was then obtained by acid hydrolysis of the starch and by fractional crystallization of the soluble sugars as described by

TABLE I

History of Diabetic Rats

Rat No	Route of alloxan administration	Duration of diabetes before experiment	Sugar excretion: Maximum per 24 hrs	Sugar excretion: During experiment		Weight: Before alloxan injection	Weight: On day of experiment	Weight: Minimum during diabetic state	Average volume of urine per day
		days	*gm*	*hrs*	*gm*	*gm*	*gm*	*gm*	*cc*
D3	Intraperitoneal	22	6 8	6	1 13	195	158	124	60
D6	"	16	5 6	6	0 36	150	150	132	50
D20	"	10	10	48	18 2	300	270	228	120
D23	"	48	11	12	2 45		163	160	70
D28	"	70	7 6		7 6*	218	212	208	50
D40	Intravenous	14	8 4	12	4 20	190	174	158	50
D48	"	25	6 8		6 6*	168	160	152	60
D53	"	20	11 3		11 3*	262	186	186	50
DJ2	"	14	11 4	46	7 0	200	164	129	65

* Rats so designated were nephrectomized, the amounts recorded were excreted during the 24 hours preceding nephrectomy

Putman *et al* (6) The purified radioglucose was dissolved in an isotonic NaCl solution and kept frozen (−18°) until just before it was injected

Collection and Determination of Exhaled CO_2—During the experiment the rat was kept in an all-glass cage which was ventilated continuously with CO_2-free air at 27–28° The air collected from the cage was passed through a column of carbonate-free NaOH (40 milliequivalents were used for each hour of CO_2 collection per rat) A porous glass disk at the bottom of the column served to break the stream of air into fine bubbles The apparatus was tested for complete recovery of the expired CO_2 The rats had access to food and water at all times while in the cage

[1] Kaplan, A , unpublished observations

[2] We are indebted to Dr W Z Hassid for the samples of photosynthetically prepared radioglucose used in this study

The NaOH-Na_2CO_3 solution was made to volume and the amount of carbonate determined by titration of two different aliquots with 0 1 N HCl One aliquot was titrated to a brom-cresol green end-point The value so obtained is a measure of the amounts of NaOH and Na_2CO_3 present To the other aliquot an excess of $BaCl_2$ was added before titrating it to the phenolphthalein end-point, this titration value represents the amount of unused NaOH The difference between the two titration values, therefore, gives the amount of Na_2CO_3 that was formed during the collection of CO_2 After centrifugation, the $BaCO_3$ precipitate was washed twice with distilled water and suspended in alcohol The precipitate was ground in a glass homogenizing tube, mounted on an aluminum disk, and its radioactivity determined after the manner described by Dauben *et al* (7) In general, each sample was counted for more than 3000 counts, the error of the counting was less than 2 per cent

Determination of Radioglucose in Urine Fermentation—A sample of urine buffered with phosphate at about pH 5 was incubated at 37° with yeast (*Torula monosa*) The yield of CO_2 evolved was improved by the presence of 10^{-3} M sodium azide (8) The carbon dioxide was collected in 0 25 N barium hydroxide solution containing 2 per cent $BaCl_2$ according to Van Slyke and Folch (9) The precipitate of barium carbonate which formed was treated as described for respiratory CO_2 The total amount of C^{14}-labeled glucose excreted in urine was obtained from the specific activity of its barium carbonate precipitate and the total urinary glucose from its reducing value

The validity of the fermentation procedure was tested on a sample of 3,4-C^{14}-labeled glucose This glucose was prepared by a modification of the method of Solomon *et al* (10) as follows A 200 gm rat was fasted for 24 hours 340 mg of Na lactate[3] were administered by stomach tube 1 hour later the animal was injected intraperitoneally with 1 millicurie of $NaHC^{14}O_3$ contained in isotonic $NaHCO_3$ 2 hours after the administration of the labeled $NaHCO_3$ the animal was sacrificed and glycogen isolated from its liver (13) Glucose was obtained by hydrolyzing the isolated glycogen for 3 hours with 0 6 N HCl

According to Wood *et al* (14) all the C^{14} atoms in this glucose are in the 3 and 4 positions Our finding that the specific activity of the $C^{14}O_2$ produced by fermentation was 3 times as high as that of the CO_2 when the whole glucose molecule was oxidized seems adequate evidence that the fermentation with *Torula monosa* is a reliable method for the determination

[3] The amount of lactate administered was based on earlier observations of Cori and Cori dealing with liver glycogen formation from lactic acid (11) It was found in the laboratory that during the first 6 hours glycogen is deposited at a uniform rate in the liver of the 24 hour fasted rat fed lactate Liver glycogen was determined by the method of van Wagtendonk (12)

of the specific activity of both carbon atoms 3 and 4 of the glucose molecule

Isolation of Osazone—The glucose content of a sample of urine was determined, and for each gm of glucose present 10 gm of phenylhydrazine hydrochloride and 15 gm of sodium acetate trihydrate were added The mixture was heated on a steam bath for 1 5 hours and then kept at 4° for approximately 12 hours The mixture was centrifuged, and the osazone crystals obtained were washed several times with distilled water The crystals were next dissolved in hot 50 per cent alcohol, and insoluble materials present removed by filtration through a steam jacket Buchner funnel The glucosazones were allowed to crystallize at 4° and then dried in a vacuum desiccator over $CaCl_2$

The glucosazones were oxidized by heating them with a chromic-sulfuric acid mixture, according to Van Slyke and Folch (9), and the carbon dioxide evolved was trapped in a mixture of 0 25 N barium hydroxide and 2 per cent barium chloride and treated as described above 92 to 93 per cent of the osazone carbon was recovered as barium carbonate The barium carbonate was mounted on an aluminum disk as described above, and its radioactivity measured The measurement of the radioactivity of the glucose is much more tedious by this method than by the fermentation procedure Furthermore, the counts per mg of $BaCO_3$ obtained by the osazone procedure suffer a 3-fold dilution by the phenylhydrazine carbon

Tables III and IV show that the values obtained for radioglucose by the two methods (osazone and fermentation) are in fairly good agreement This agreement implies that the specific activity of the 3rd and 4th carbons of the glucose molecule is approximately equal to that of the other carbons This is important since it excludes the possibility that the expired $C^{14}O_2$ is derived only from one or two specific carbons of the glucose chain

Results

Conversion of Radioglucose to CO_2 by Normal and Diabetic Rats

Normal Rats—Rats N1 and N2 received intraperitoneally 100 mg of radioglucose (Table II), whereas Rats 3 to 5 were injected intraperitoneally with 1 gm of labeled glucose per kilo of body weight The amounts of radioactive CO_2 recovered in the expired air of each rat are recorded in Table II During the first 4 hours, the administered labeled glucose was rapidly oxidized by the normal rat By the time 6 hours had elapsed, 40 to 60 per cent of the administered C^{14} was recovered in the expired CO_2 Although in 6 hours Rat N2 eliminated as CO_2 as much as 55 per cent of the radioglucose that it received, only an additional 11 per cent was recovered as expired radioactive CO_2 during the next 18 hours The more rapid conversion of the administered labeled glucose to CO_2 during the early intervals

means, of course, that at these times the animal's labeled glucose pool had a high specific activity and that at the later intervals this had been diluted by unlabeled glucose derived either from the diet or from endogenous sources

Diabetic Rats—At the time radioactive glucose was injected, diabetes had existed in the animals used for periods varying from 10 to 48 days (Table I) The rats displayed such manifestations of diabetes as polyuria, glycosuria, polyphagia, and weight loss Each rat excreted from 50 to 120 cc of urine per day The amount of glucose excreted was, of course, not constant from day to day The maximum found before the administration of radioglucose amounted to 11 gm per 24 hours

TABLE II

*Oxidation of Intraperitoneally Injected Radioglucose to CO_2 by Normal Rats**

Rat No	Weight	Radioglucose injected intraperitoneally	Per cent of administered glucose† converted to CO_2 at end of				
			2 hrs	4 hrs	6 hrs	12 hrs	24 hrs
	gm	*mg*					
N1	172	100			56		
N2	250	100	27 6	48 4	55	61 6	65 7
N3	230	230‡			56 7	63 3	
N4	270	270‡			45 9	53 6	
N5	280	280‡			39 4	49 6	

* The rats had access to food throughout the period of observation

† The administered glucose contained 1 to 2 million counts per minute

‡ Equivalent to 1 gm per kilo of body weight

In contrast to the normal rat, which had converted approximately 40 to 60 per cent of the administered glucose to CO_2 in 6 hours, the five diabetic rats used in this study (Tables III and IV) oxidized only 11 to 24 per cent of the injected labeled glucose in 6 hours An examination of the urine revealed, however, that most of the administered radioglucose was not available for oxidation but had been excreted Thus Rat D20 excreted more than 60 per cent of the administered glucose in the first 12 hours after its injection (Table III) Little more radioglucose was excreted in the next 36 hours This does not mean that the rat did not continue excreting glucose, but rather that after 12 hours the specific activity of the body glucose had dropped to low levels In 6 hours the other four rats (Table IV) excreted from 30 to 45 per cent of the labeled glucose they had received intraperitoneally

Calculation of Rate of Glucose Oxidation

From the amount of $C^{14}O_2$ expired during a given interval after the injection of the radioglucose, it is possible to obtain an approximate value for the amount of plasma glucose that had been oxidized during that interval

TABLE III

Oxidation of 100 Mg of Intraperitoneally Injected Radioglucose by Diabetic Rat D20

Interval	Total glucose	Radioglucose, per cent of injected dose determined by		Specific activity* of urinary glucose × 10³	Expired $C^{14}O_2$, per cent of administered glucose†	Glucose oxidized
		Fermentation	Osazone			
hrs	*mg*					*mg per hr*
0– 2	775	49 3	43 1	60	6 6	55
2– 4	513	11 0	9 7	20	3 3	82
4– 6					1 5	
4–12	2,260	3 3	5 0	1 9		
6–12					2 1	
12–24	5,350	0 7‡	‡	0 13	1 0	
24–48	9,300	‡	‡		0 7‡	
Total	18,200	64 3			15 2	

* The specific activity is expressed as the per cent of the injected C^{14} per mg of glucose The values are based on averages between fermentation and osazone

† The injected dose contained 1,800,000 counts per minute

‡ The sample counted less than 1 2 × background

TABLE IV

Oxidation of Intraperitoneally Injected Glucose by Diabetic Rats D3, D6, D23, and D40

Rat No	Weight	Radioglucose injected	Urinary glucose: Total		Urinary glucose: Radioglucose per cent of injected dose* determined by: Fermentation		Osazone		Specific activity† of urinary glucose × 10³		Expired $C^{14}O_2$, per cent of administered glucose*		Glucose oxidized‡
			1st 6 hrs	2nd 6 hrs	1st 6 hrs	2nd 6 hrs	1st 6 hrs	2nd 6 hrs	1st 6 hrs	2nd 6 hrs	1st 6 hrs	2nd 6 hrs	
	gm	*mg*	*mg*	*mg*									*mg per hr*
D3	158	100	1130		34		36 8		31		23		122
D6	150	100	360		46		44 5		125		24		32
D23	163	163§	379	2070	30	13 5	30 5	10 9	79	6 0	15	3 7	32
D40	174	174§	1360	2840			45 7	3 0	34	1 1	17 5	1 8	87

* The administered glucose contained 1 to 2 million counts per minute

† The specific activity is expressed as the per cent of the administered C^{14} per mg of glucose

‡ These values are determined from the specific activity of the urinary glucose and *the expired $C^{14}O_2$ during the first 6 hours of the experiment*

§ The glucose injected equals 1 gm per kilo of body weight

To make such a calculation, we used the *average* specific activity of plasma glucose during the period under consideration But we have to be

reasonably certain that the expired $C^{14}O_2$ is derived only from labeled glucose, as a rule, this will be true only during the early intervals after the injection of radioglucose when little or none of it has been converted to other compounds

To determine the specific activity of plasma glucose, an aliquot of the plasma sample was oxidized and its total radioactivity determined as $BaCO_3$, whereas on a different aliquot of this same plasma the glucose content was determined from its reducing value In the diabetic animals we were able to check this procedure by demonstrating that the specific activity so obtained was equal to the specific activity of the urinary glucose This is shown in Fig 1

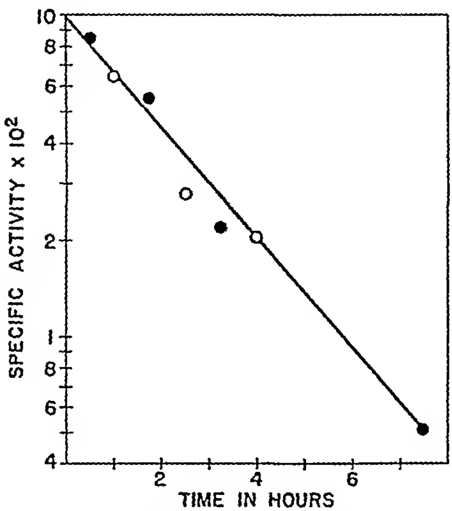

Fig 1 Changes with time in specific activity of urinary and plasma glucose of Rat DJ2 The ordinate scale is logarithmic O plasma glucose, ● urine glucose

Values for specific activities of plasma and urinary glucose of two diabetic rats (Rats DJ2 and D20) are presented in Figs 1 and 2, here the values are plotted against time on semilog paper This plot was found to be a straight line during the first 6 hours (15) During the interval from 0 to 2 5 hours the average specific activity of plasma glucose, expressed as a percentage of the injected dose per mg of glucose, is approximately 6×10^{-2} (see Fig 1) The amount of $C^{14}O_2$ exhaled during that interval was approximately 6 per cent of the administered radioactivity Therefore, the amount of glucose oxidized in this period is equal to $6/(6 \times 10^{-2}) = 100$ mg or 40 mg per hour The same calculation for the interval from 2 5 to 4 hours, in which 2 per cent of the injected dose was exhaled, gives an oxidation of 50 mg of glucose per hour

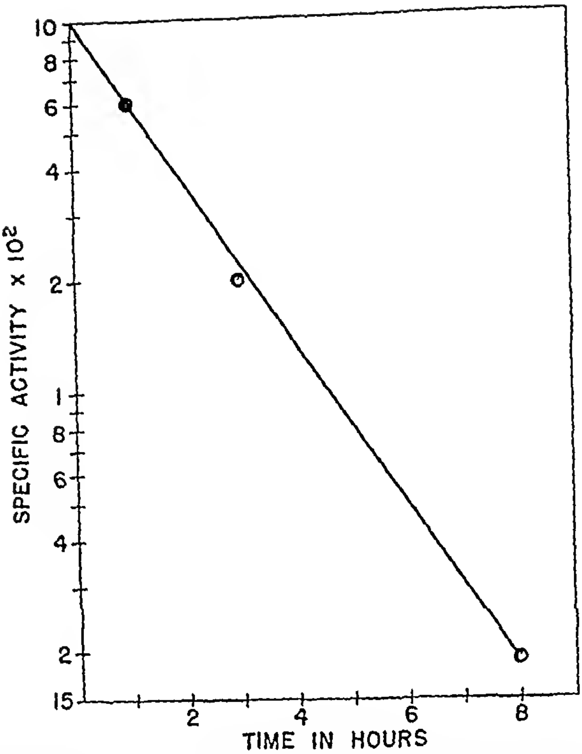

FIG 2 Changes with time in specific activity of urinary glucose of Rat D20 (see Table III)

TABLE V

Rate of Oxidation of Glucose by Normal Rats

Each rat was injected intraperitoneally with 15 mg of labeled glucose

Rat No	Weight	$C^{14}O_2$ collected		Plasma glucose		Glucose oxidized
		Interval	Per cent of injected dose*	Time after injection	Specific activity† × 10²	
	gm	*hrs*		*hrs*		*mg per hr*
NJ1	220	0–1	13 6		‡	50
		1–2	10 6	1	20 8	66
		2–4	5 5	3	6 9	40
		4–6	1 84	6	4 6	
NJ2	242	0–1	8 75		‡	32
		1–2	9 0	1	19	69
		2–4	5 25	3	4 1	64
		4–6	2 73	6	4 1	
NJ3	250	0–1	7 93		‡	28
		1–2	14 1	1	21 8	85
		2–3	4 63	3	7 6	48

* The injected glucose contained 1,600,000 counts per minute

† The specific activity is expressed as the percentage of the injected C^{14} per mg of glucose

‡ The average specific activity of plasma glucose was obtained by extrapolation (see the text)

The values obtained by this type of calculation for the amounts of glucose oxidized per hour by normal rats are recorded in Table V and by diabetic rats in Tables III and IV The range of values calculated for the normal rat was similar to that found for the diabetic

Comparison of Ability of Nephrectomized Normal and Nephrectomized Alloxan-Diabetic Rats to Convert Radioglucose to CO_2

Since the diabetic rat excreted a good portion of the administered glucose in the urine, the amount of $C^{14}O_2$ exhaled, as noted above, provided no basis for comparing the capacities of normal and diabetic rats to oxidize administered glucose To prevent loss of glucose by this route, radio-

TABLE VI

Conversion of Intravenously Injected Radioglucose to CO_2 by Nephrectomized Normal and Nephrectomized Alloxan-Diabetic Rats

Each rat received 1 gm of labeled glucose per kilo of body weight

Rat No		Weight	Per cent of administered glucose* recovered as CO_2 for each interval						Blood sugar at end of experiment
			0-0 5 hr	0 5-1 hr	1-2 hrs	2-4 hrs	4-6 hrs	Entire 6 hrs	
		gm							mg per cent
Normal	N6	180	5 7	5 3	8 0	14 0	3 9	36 9	131
"	N7	172	2 7	4 7	6 0	12 3	3 9	29 6	157
"	N8	202	3 9	4 4	8 7	15 6	3 3	35 9	125
"	N9	174	5 6	4 8	8 5	16 7	3 6	39 2	138
Diabetic	D28	212	0 42	1 4	4 5	10 5	9 4	26 2	700
"	D48	160	2 2	5 9	10 0	13 7	7 8	39 6	870
"	D53	186	0 57	2 1	4 3	11 4	7 5	25 9	1060

* The administered glucose contained 1 to 2 million counts per minute

glucose was injected intravenously into rats 15 minutes after they had been subjected to bilateral nephrectomy Each rat received 1 gm of glucose per kilo of body weight This dose was selected because it was large enough to yield similar *initial* values for the specific activities of circulating glucose in both normal and diabetic rats In other words, this dose minimized the differences in plasma glucose between the normal and the diabetic rats at the very beginning of the experiment Data on the conversion of injected radioglucose to $C^{14}O_2$ by the two types of rats under these conditions are recorded in Table VI

Judging from the $C^{14}O_2$ exhaled during the entire 6 hours, there appears to be no significant difference between nephrectomized normal and nephrectomized alloxan-diabetic rats in their ability to oxidize the administered glucose It is interesting to note, however, that in the early intervals

two of the three diabetic rats exhaled less $C^{14}O_2$ than the normal ones, whereas at the later intervals (4 to 6 hours) the reverse was the case

6 hours after the administration of the large dose of labeled glucose, namely 1 gm per kilo of body weight, the plasma glucose in the nephrectomized normal rats had returned to normal levels or levels close to normal, the highest value observed at the 6 hour interval was 160 mg per cent In the diabetic rats, on the other hand, plasma glucose of 700 to 1000 mg per cent was found when they were sacrificed

DISCUSSION

Normal rats injected intraperitoneally or intravenously with amounts of glucose varying from 100 to 280 mg converted 40 to 50 per cent of it to CO_2 in 6 hours In the nephrectomized but otherwise normal rat, $C^{14}O_2$ appeared in the exhaled gas within the first half hour, and at the end of the 1st hour nearly 10 per cent of the intravenously injected glucose was converted to CO_2 These findings are in agreement with the classical view that administered glucose is available for immediate oxidation by the animal body

In order to compare the rates of *conversion* of *plasma* glucose to CO_2 by normal and diabetic rats, two methods of study were employed one was based on the $C^{14}O_2$ evolved when a relatively large amount of labeled glucose was introduced intravenously into nephrectomized preparation, in the second the amount of glucose oxidized in the intact rat was derived from measurements of the $C^{14}O_2$ evolved and of the specific activity of plasma glucose That the alloxan-diabetic rat can oxidize appreciable amounts of glucose cannot be doubted from the results obtained by the first method As judged by the $C^{14}O_2$ eliminated during an entire 6 hour period of observation, the amounts of administered glucose oxidized by the nephrectomized normal and nephrectomized diabetic rats are of the same order *The values for glucose oxidation obtained by the second method refer specifically to the conversion of plasma glucose (and such glucose as exists in rapid equilibrium with plasma glucose) to CO_2*

The interpretation of our findings is not without some difficulty The values for specific activity of plasma glucose are based on two measurements, namely, (1) the total radioactivity of plasma, which we have assumed to be glucose at the early intervals, and (2) the total reducing value of plasma, which we have also assumed to be glucose The reliability of the specific activity measurements for plasma glucose is shown by the finding that in the diabetic rat the values obtained agreed with those found for the osazones prepared from urinary glucose In the normal rat it was not possible to make such a comparison The assumptions made in connection with the specific activity would appear, however, to be well supported by our

observations in the nephrectomized rats, the finding that the over-all capacity of the normal nephrectomized rat to oxidize glucose is of the same order as that of the diabetic involves none of the assumptions referred to above

An interference was observed in the oxidation of the injected carbohydrate by the nephrectomized diabetic rat during the 1st hour, whereas at the later intervals the amount of $C^{14}O_2$ produced by these rats was of the same order of magnitude as that produced by normal rats The interpretation of the results of the early intervals presents some difficulties This phase is being investigated further

In order to interpret the $C^{14}O_2$ data presented here for the alloxan-treated rats, the degree of diabetes that existed in these rats must be evaluated Postmortem examination of their pancreases revealed massive necrosis of the islets It cannot be inferred, however, that the alloxan-diabetic rat is deprived of *all* insulin-secreting tissue But even though the presence of some residual insulin cannot be excluded in the diabetic rats used in this study, they nevertheless excreted, when fed, from 5 to 11 gm of glucose daily and exhibited such manifestations of diabetes as polyphagia, polyuria, polydipsia, and loss of weight Another indication of the degree of diabetes in the rats used here is provided by the finding of values for plasma glucose of 700 to 1000 mg per cent 6 hours after excision of their kidneys

SUMMARY

1 The rate of conversion of administered radioglucose to CO_2 was measured in normal and alloxan-diabetic rats

2 $C^{14}O_2$ appeared within the first 30 minutes in the expired air of both normal and alloxan-diabetic rats

3 The amount of *plasma* glucose converted to CO_2 was measured by two methods The first was based on the specific activities of plasma glucose and the amounts of $C^{14}O_2$ in the expired air, the second on the amounts of $C^{14}O_2$ exhaled by the nephrectomized rat As judged by these procedures, the rate of conversion of *plasma glucose* to CO_2 by the alloxan-diabetic rat does not differ significantly from that found in the normal

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PHYSICOCHEMICAL PROPERTIES OF CRYSTALLINE CLOSTRIDIUM BOTULINUM TYPE A TOXIN*

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Interest in the chemical nature and mode of action of bacterial toxins has been renewed by the simultaneously announced crystallization of botulinal (1) and tetanal toxins (2) and the recent purification of several toxoids (3–5) Of these, crystalline *Clostridium botulinum* type A toxin alone has been submitted to an apparently complete elementary and amino acid analysis (6), as well as to preliminary physicochemical characterization (7–9) and immunological study (10) This toxin has been found to be a typical protein exhibiting no unique composition or physicochemical properties and is apparently devoid of a prosthetic group A preliminary report of the molecular weight and homogeneity of botulinal toxin prepared by the method of Lamanna *et al* (1) has already been made (9) Further details of the physicochemical characterization of this toxin and of the effect of the method of preparation on the molecular kinetic and serological properties of this substance are given in this report Investigation of the botulinal toxin-antitoxin reaction has also been undertaken Data to be published separately indicate that serologically the toxin acts as a single substance (10)

The original procedure for the crystallization of botulinal toxin (1) has been modified in several ways, including the omission of a step involving shaking of the crude acid-precipitated toxin with chloroform (11) However, much study had already been made of the properties of crystalline toxin of maximum potency prepared by the procedure first announced A later method described by Abrams, Kegeles, and Hottle (7) differs from both the above procedures and also omits shaking with chloroform In view of the suggestion (8) that partial denaturation may result from the use of chloroform in the purification of the toxin, some data on the physicochemical and serological characteristics of toxin prepared both by the original and the modified methods of Lamanna *et al* are included in this communication

* Some of the material in this paper was presented at the meeting of the Federation of American Societies for Experimental Biology at Chicago, May, 1947 (*Federation Proc* , 6, 284 (1947))

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EXPERIMENTAL

The crystalline toxin used in this investigation was prepared by method previously described (1, 11) Some of the material was later used for the amino acid analysis already mentioned (6) Several lots were studied, including one not subjected to shaking with chloroform (Batch S-5) The potency of the toxin varied somewhat from preparation to preparation but probably not significantly outside the errors of the mouse titration employed The toxin contained about 220 × 10^6 mouse LD_{50} per mg of N as measured by a statistically valid titration (12) on 20 gm white mice The highest value recorded was 250 × 10^6 LD_{50} per mg of N (initial potency of Batch C) A single batch of toxin (Batch BT-3), characterized successively in electrophoresis, diffusion, and sedimentation behavior retained a potency of 170 × 10^6 LD_{50} per mg of N upon recovery It was observed, however, that even mild physical treatment of the purified toxin resulted in some loss of toxicity, especially at high dilutions

Electrophoresis

The electrophoretic homogeneity of botulinal toxin was studied with the aid of the Tiselius apparatus (13) One apparatus used was equipped with the Philpot-Svensson optical system (14), another employed in later experiments utilized the Longsworth scanning system (15) All physical measurements were made on preparations equilibrated by long dialysis against buffer All pH measurements were made with the glass electrode

Toxin of maximum potency prepared by shaking with chloroform is homogeneous in electrophoresis, as is illustrated by the diagrams of Fig 1 However, the relatively small amounts of purified toxin available restricted the scope of these experiments In this instance, only the lower middle section and bottom section of the two-compartment cell could be filled with the protein solution In Fig 1, photographs[1] at two time intervals are given to illustrate the low degree of boundary spread with time The upper diagrams represent migration for 11,400 seconds, the lower for 14,400 seconds The mobility at 1° in 0 1 N sodium acetate buffer, pH 4 38, calculated from the diagrams of Fig 1, is 2 75 × 10^{-5} cm ^{2} volt^{-1} sec $^{-1}$

No indication of a component other than toxin is to be seen in Fig 1 It was regularly found that toxin freshly crystallized by the original method of Lamanna and coworkers (1) was electrophoretically homogeneous under these conditions Occasionally, a small, fast moving shoulder was observed on the descending boundary, but this could always be removed by repeated crystallization On long standing, one preparation (Batch C) yielded a sharp single ascending boundary of normal mobility but a sp' '

[1] The initial boundaries are slightly obscured because of light absorption by detoxifying agent added to the external constant temperature bath

descending boundary However, at this time the preparation also appeared somewhat inhomogeneous, as judged both from diffusion studies and from light absorption photographs in the ultracentrifuge On conversion to toxoid by treatment with formalin, the electrophoretic diagrams of this

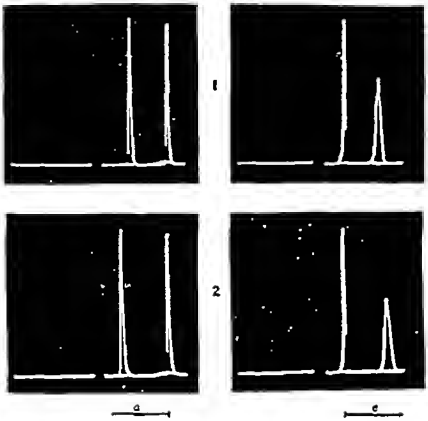

FIG 1 Electrophoretic patterns of crystalline botulinal toxin prepared *with chloroform shaking*, pH 4 38, ionic strength 0 1, 0 1 N sodium acetate 0 2 N acetic acid buffer Photographs taken by the Philpot-Svensson method after migration at 1° at a field strength of 5 32 volts per cm in the two-section cell Upper photograph, 11,400 seconds, lower photograph, 14,400 seconds The arrow indicates the direction of migration, *a* and *d* represent the ascending and descending boundaries respectively The very steep curves at the tails of the arrows are superimposed photographs of the starting boundaries

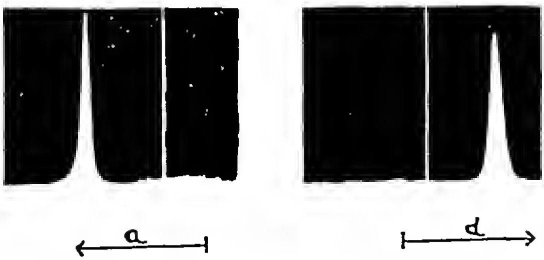

FIG 2 Electrophoretic patterns of crystalline botulinal toxin prepared *without chloroform shaking* Photographs taken by the Longsworth scanning method after 12,300 seconds migration at 5 37 volts per cm Conditions and symbols as in Fig 1

material were unaltered, although the mobility on the alkaline side of the isoelectric point was increased

Crystalline toxin, prepared by the modified method of Lamanna and coworkers (11) with the step of shaking with chloroform omitted, was likewise homogeneous in electrophoresis, as shown in Fig 2 The mobility

at pH 4 38 was 2 69 × 10^{-5} cm ^{2} volt^{-1} sec $^{-1}$, the same within experime error as for toxin prepared with chloroform shaking

The small degree of boundary spread observed for both types of arations indicated that the toxin was quite homogeneous in electropho. Since there was some question that the chloroform treatment may altered the toxin, the boundary spread for the two lots was analyzed tistically by a method already described (16) Unfortunately, th solubility at the isoelectric point precluded boundary spread analy that pH range, the region best suited to this study For a single mov boundary, the procedure of Sharp *et al* (16) yields a value, denoted as heterogeneity constant, which has the dimensions of mobility and increa with the electrical heterogeneity of the protein The values for the preparations compared favorably, being 1 4 × 10^{-6} cm ^{2} volt^{-1} sec $^{-1}$ the salt-fractionated toxin, and 1 8 × 10^{-6} cm ^{2} volt^{-1} sec $^{-1}$ for toxin pr pared by shaking with chloroform These figures indicate a relatively degree of reversible boundary spread (*i e*, spread not attributable to fusion alone)

Diffusion

The diffusion constant (D) of crystalline toxin, previously found to homogeneous in electrophoresis, was determined at 25° by the refractr index scale method of Lamm (17), in the Neurath cell and apparatus (' The values are given in Table I The subscripts on D indicate the sev methods of calculation which allow different weight to various factors (1. For the first time interval given (84,300 seconds, or approximately 1 (the boundary was yet too sharp for accurate measurement of the scale i displacements With this exception, there is excellent agreement b among the values found at different time intervals and among those tained at a given time interval when calculated by the several metho The latter finding is a good criterion of molecular kinetic homogeneity, measured by the somewhat insensitive method of diffusion, and may be e pressed numerically by the Gralén index (19) given in the last column Table I as $D_{\sigma}/D_A{}^2$ The deviation of this constant from unity is a measu of the distribution of sizes in a substance

Similar evidence for homogeneity in diffusion behavior is offered by t low value of the standard deviation for the diffusion constant calculated points along the curves by the method of successive analysis (the six column, Table I) However, the best criterion of homogeneity in diffusi lies in transforming the actual diffusion curve to normal coordinates a making graphical comparison to the ideal Gaussian distribution curv The rather good fit obtained by this procedure and the close merging of t maximum ordinates of the ideal and the calculated curves is shown in Fi 3 (time, 354,840 seconds)

Sedimentation in Ultracentrifuge

The sedimentation characteristics of the crystalline toxin were studied in air-driven analytical ultracentrifuge (20), the rotor of which carried a sector-shaped cell at a mean radius of 6 5 cm The ultraviolet light absorption method was employed for preliminary analysis, the Lamm scale method for quantitative studies Sedimentation velocity scale diagrams obtained at a mean temperature of 32° and a mean centrifugal field of 00g have already been published (9) The diagrams revealed a single sharp sedimenting boundary and yielded a value for the sedimentation

TABLE I

Diffusion Constant of Crystalline Botulinal Toxin† at 25°*

Time	D_M	D_A	D_{A^2}	D_σ	D_S	$\frac{D_\sigma}{D_{A^2}}$
sec	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	
84,300	2 08	2 10	2 12	2 19	2 05 ± 0 06‡	1 03
39,480	1 99	1 96	1 96	1 97	2 06 ± 0 07	1 00
74,860	1 96	1 95	1 95	2 01	2 05 ± 0 12	1 03
354,840	1 98	1 94	1 89	1 95	2 04 ± 0 05	1 03
Average	2 01 ± 0 07 × 10^{-7} cm ^{2} sec $^{-1}$					
D_{25}	2 14 × 10^{-7} cm ^{2} sec $^{-1}$					
D_{20}	1 87 × 10^{-7} cm ^{2} sec $^{-1}$					

* D_M, D_A, D_{A^2}, D_σ, and D_S denote, respectively, the diffusion constant (with the dimensions cm ^{2} sec $^{-1}$) calculated by the maximum height, maximum height area (squared), maximum height-area (squared), standard deviation, and successive analysis methods (18) D_{25} and D_{20} denote the diffusion constant corrected to the water basis at the respective temperatures D_{20} is given to permit comparison with the average value 2 02 × 10^{-7} cm ^{2} sec $^{-1}$, determined independently in the Tiselius apparatus by the Longsworth method on other batches prepared by both groups of workers (8)

† Protein concentration of 0 63 per cent in 0 1 N sodium acetate buffer, pH 4 38

‡ Standard deviation from the mean of determinations at six or more points along the curve

constant, S_{20}, of 17 3 Svedberg units at a protein concentration of 0 17 per cent, the lowest concentration studied Although the base-lines of the sedimentation diagrams exhibited a small angular deviation from the horizontal, planimetric measurement of the area under each boundary revealed a constant concentration of sedimenting substance equal to the initial concentration (calculation by Equation 183a (21), including a correction for

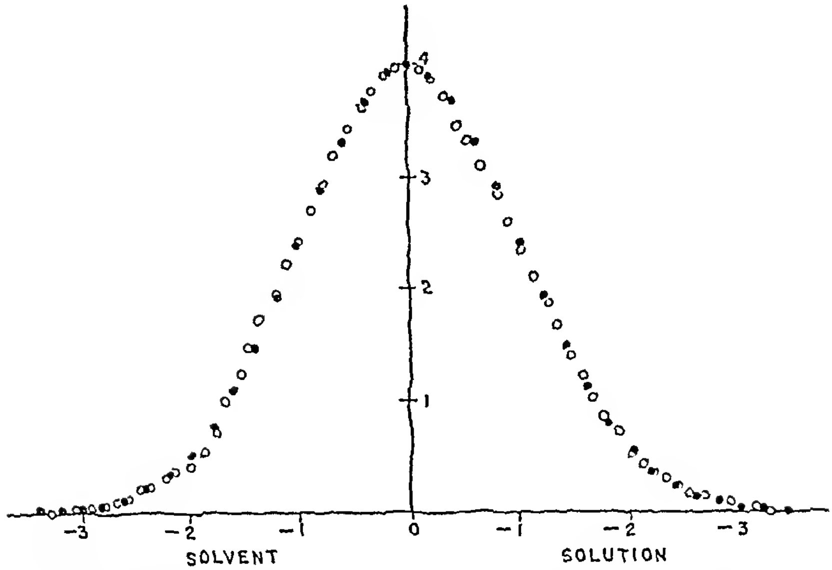

FIG 3 Comparison of an ideal Gaussian distribution curve with the diffusion curve obtained on a 0 63 per cent solution of crystalline botulinal toxin ● indicates the position of the ideal curve, O represents the experimental scale line displacements, plotted in normal coordinates Time, 354,840 seconds

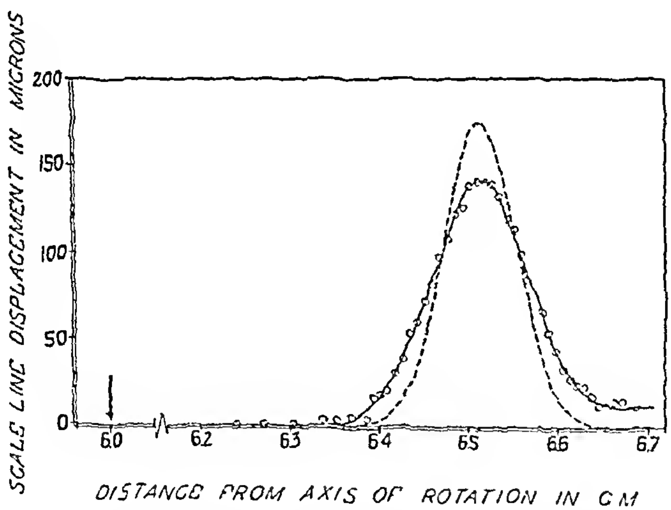

FIG 4 Boundary spreading in a sedimentation experiment with crystalline botulinal toxin The solid line indicates actual points obtained with the Lamm scale method, the dash line represents theoretical *spreading due to diffusion alone* Time, 5400 seconds at 48,000*g*

of the toxin The results are shown in Fig 4, which gives the scale diagram for Batch BT-3 after 5400 seconds sedimentation at a speed of 25,400 R P M ...ing ... line represents ... ison ...ual scale diagram, the dash line depicts the The rather good ... ...med by this procedure and ... *maximum ordinates of the ideal and the calculated* (21)), with the diffusion 3 (time, 354,840 seconds)

constant obtained in separate diffusion experiments (see above) It may be seen that the boundary spread is somewhat greater than that attributable to diffusion alone This is confirmed by the fact that the apparent diffusion constant calculated from the sedimentation diagrams, though variable, is greater than that obtained from the separate diffusion experiments

The extraboundary blurring which occurs during the sedimentation of botulinal toxin may have been due to experimental conditions, such as possible slight vibration of the rotor or the difficulty of establishing the zero time of diffusion, or it may be indicative of a small degree of molecular heterogeneity However, inspection of Fig 4 reveals that the distribution of sedimentation rates does not differ significantly from the mean rate If the increased boundary spreading is attributed to molecular heterogeneity, it could have originated either from a population of molecules differing but slightly in molecular size or from the presence of another substance of closely similar sedimentation properties It was our experience that even mild physical procedures for study of this toxin induced some loss of potency, possibly by toxoid formation through surface denaturation We thus attribute to this effect the small observed deviation of the actual sedimentation diagrams from theoretical curves calculated from diffusion data

Molecular Weight and Shape

The sedimentation constant and diffusion constant taken together permit the calculation of the molecular frictional ratio $(f/f_0)_s$ according to the Perrin theory (*cf* pp 41–43 (21)), yielding a value of $(f/f_0)_s$ of 1 76 On the assumption that the molecules resemble prolate ellipsoids, this figure corresponds to a ratio of major to minor axis (b/a) of 14 6, hydration being neglected (21) Substitution of the sedimentation constant and the diffusion constant in the Svedberg equation (Equation 3a (21)), with a value of 0 75 (8) for the partial specific volume at 20°, yields a molecular weight of 900,000 [2]

In an effort to ascertain the particle size and shape of botulinal toxin directly, a few attempts were made to photograph a preparation shadowed with gold, by the use of the RCA electron microscope Because of the small size of the toxin, relative to viruses and other biological substances usually studied in this apparatus, the results were inconclusive It is hoped that

[2] The partial specific volume of chloroform fractionated botulinal toxin calculated from the complete amino acid data is 0 736 This figure based on tentative values for the constituent amino acid molar volumes leads to a slightly lower molecular weight (Cohn, E J, and Edsall, J T, Proteins, amino acids and peptides, American Chemical Society monograph series, New York, 370–375 (1943))

resort to the newer techniques for the production of metal shadow coatings, with platinum or palladium on glass with subsequent replica stripping, will yield more decisive results in the near future

Immunological Homogeneity

In addition to meeting the physicochemical criteria of homogeneity in electrophoresis and diffusion and sedimenting with a single sharp boundary in the ultracentrifuge, botulinal toxin has proved to be homogeneous in immunological behavior, for both toxin and antitoxin are completely precipitated in the equivalence zone (10) Moreover, quantitative comparison of the behavior in the precipitin reaction of batches of toxin prepared with and without chloroform shaking reveals no difference as a result of this treatment When the ratio of antitoxin to toxin nitrogen in the precipitate in the zones of antibody excess and equivalence is plotted against the amount of toxin nitrogen added, according to the procedure of Heidelberger and Kendall (22), the curves for toxin prepared by the two methods coincide (*cf* Fig 5) However, for both substances a curvilinear relation obtains, even when the ratio of antitoxin to toxin nitrogen is plotted against the square root of the added toxin concentration Botulinal toxin thus differs in precipitin behavior from ordinary protein antigens which usually yield a linear relationship between the antibody to antigen ratio and the amount of added antigen A more complete discussion of the characteristics of the botulinal toxin-antitoxin reaction will be published separately (10).

DISCUSSION

Crystalline *Clostridium botulinum* type A toxin meets some of the usual criteria of protein purity, *i e* electrophoretic homogeneity, homogeneity in diffusion, sedimentation with a single boundary, and serological homogeneity in the toxin-antitoxin reaction However, the toxin fails to meet a more rigorous test of protein purity, namely sedimentation without boundary spreading greater than that solely attributable to normal diffusion In addition, preliminary phase rule solubility studies indicated the presence of more than one component The solubility studies were discontinued because surface denaturation of the toxin was readily brought about by the shaking methods used to achieve equilibrium

The apparent slight molecular inhomogeneity of botulinal toxin is attributed to the marked lability of this protein rather than to the presence of foreign components Even ordinary handling at room temperature readily induces surface denaturation of solutions, with the formation of opalescence or visible aggregates, requiring clarification by centrifuging at a low speed in the angle centrifuge prior to physicochemical study Spontaneous loss

of toxicity also occurs on long standing or at high dilution, necessitating the addition of protective agents at the extreme dilutions used in the estimation of biological potency Rapid inactivation takes place in solutions above pH 7

The lability and protein nature of highly purified bacterial toxins so far studied appears to be a general phenomenon Crystalline tetanal toxin, originally electrophoretically homogeneous and apparently a single sub-

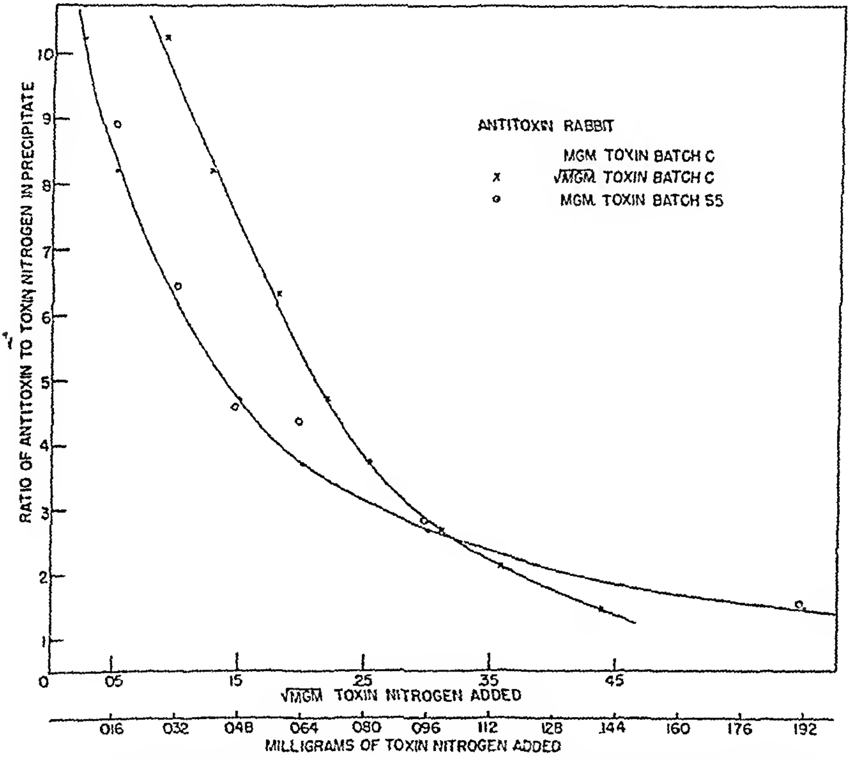

FIG 5 Composition of toxin antitoxin precipitates in the zones of equivalence and antitoxin excess Batch C was crystallized by the chloroform shaking method, Batch S 5, by a different method without shaking with chloroform

stance serologically, undergoes spontaneous detoxification on standing at 0° without loss in antitoxin-flocculating power but with marked alteration in phase rule solubility properties (23) Moreover, this toxin shows two components in the ultracentrifuge, one apparently an atoxic dimer Diphtherial toxin is essentially homogeneous by molecular kinetic criteria (24) but is rapidly inactivated above pH 6 0 (5) It has been reported that electrophoretically isolated, electrically homogeneous, erythrogenic toxin

of scarlet fever is nearly homogeneous in the ultracentrifuge as judged by comparison of the actual and theoretical sedimentation curves (25) However, the diffusion constant used in the latter analysis was not obtained by special precise diffusion experiments as in this investigation, but rather was a mean value obtained by calculation from a series of the same sedimentation curves No bacterial toxin has yet been reported to exhibit a constant solubility However, purified diphtheria toxoid does have a constant solubility and satisfies other criteria of protein purity (5)

The molecular constants for crystalline botulinal toxins obtained in this investigation are in good agreement with those previously reported Diffusion studies with the sintered glass disk technique afforded an estimate of about 1 million for the molecular weight (1) The minimum molecular weight calculated from amino acid analysis is approximately 45,000 (6), one-twentieth of the value, 900,000, determined by physical means and reported in this work The molecular weight calculated by Kegeles (8) from diffusion and viscosity measurements, with the assumption that the toxin molecules approximate elongated ellipsoids, is 1,130,000 Using different method the latter author obtained a diffusion constant for toxin prepared by the procedure of Lamanna *et al* of $D_{20} = 1\ 79 \times 10^{-7}$ cm^2 sec^{-1}, in good agreement with our value of $1\ 87 \times 10^{-7}$ cm^2 sec^{-1} (correction made to 20°) However, for preparations made by the method of Abrams *et al*, Kegeles found a mean value of $D_{20} = 2\ 13 \times 10^{-7}$ cm^2 sec^{-1} Since the ultracentrifuge and diffusion data reported in our study were obtained only on chloroform-fractionated toxin, the identity of size and shape of the toxin prepared by the several methods has not yet been clearly established

Conclusions with regard to the apparent molecular shape of this protein must await further study The frictional ratio derived from the sedimentation and diffusion constants in this investigation ($(f/f_0)_s = 1\ 76$) is markedly greater than that obtained from viscosity measurements (8) and the assumption of an elongated ellipsoidal molecular model ($(f/f_0)_\eta = 1\ 45$) In the latter study, fractions prepared by both methods were used in viscosity measurements, though one preparation subsequently proved to be electrophoretically inhomogeneous. Unless excessive hydration is assumed, neither value of the frictional ratio derived by these indirect physical methods is in accord with the report that electron micrographs of inactive formalin-treated toxin (toxoid) show particles nearly spherical in shape (11)

No explanation for the extreme toxicity of this protein can be deduced either from its composition or from its physical properties However, the finding of a high molecular weight for botulinal toxin compared to other bacterial poisons poses two difficult problems first, the explanation of the apparent absorption of the toxin through the gut in accidental botulism resulting from ingestion of spoiled foods, second, the elucidation of its mode

of action on administration by either the oral or intraperitoneal route The singular oral toxicity of this protein is in accord with reports that it is relatively resistant to the proteolytic action of pepsin and trypsin (*cf* (26))

The suggestion has been made that tetanal, botulinal, and diphtherial toxins may act by interfering with the synthesis of some essential enzyme, and some evidence has been presented indicating that diphtherial toxin perhaps blocks the synthesis of cytochrome *b* or some closely related enzyme (26) However, botulinal toxin is far more potent than diphtherial toxin (26), and it may be calculated that on the basis of a molecular weight of 900,000 only 20,000,000 molecules are required to kill a mouse This fact, together with the protein nature of the toxin, suggests that it may attain its potency indirectly by acting enzymatically to synthesize a cellular poison from some normal metabolite or otherwise break an indispensable link in some physiological reaction chain in nervous tissue

The authors are indebted to Dr Hans Neurath of the Department of Biochemistry, Duke University School of Medicine, for making available to one of us the diffusion apparatus used in this study and to Dr Gerson Kegeles, National Cancer Institute, for suggestions on the manuscript

SUMMARY

1 Crystalline type A botulinal toxin is homogeneous in electrophoresis and in diffusion

2 The toxin sediments with a single sharp boundary in the ultracentrifuge but with spreading somewhat greater than that attributable solely to diffusion This behavior may indicate slight molecular heterogeneity attributable to the lability of this protein

3 The molecular weight obtained from sedimentation and diffusion data is 900,000, the apparent frictional ratio is 1 76

4 Quantitative precipitin studies indicate that botulinal toxin is serologically a single substance The ratio of antibody to toxin in the precipitates in the zones of antibody excess and equivalence is not linearly related to the amount of antigen added

5 Crystalline toxin prepared by several methods possesses identical electrophoretic and serological properties

6 Its extreme potency, high molecular weight, and protein nature suggest that botulinal toxin may be an enzyme However, no explanation of the mode of its pharmacological action is afforded by these studies

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XANTHOMYCINS A AND B, NEW ANTIBIOTICS PRODUCED BY A SPECIES OF STREPTOMYCES*

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Two new antibiotics have been obtained from culture filtrates of an unidentified species of *Streptomyces* These antibiotics are of special interest because of their high potency and their extreme toxicity Because they are yellow in color and are produced by a *Streptomyces*, they have been named xanthomycin A and xanthomycin B

The organism, designated *Streptomyces* 94, is one of a series isolated from oats, flax, and wheat Because *Streptomyces* 94 had shown marked antagonistic activity against both Gram-positive and Gram-negative bacteria in a preliminary agar streak test, it was chosen for further study

EXPERIMENTAL

Antibiotic Production by Streptomyces 94

Method of Assay—In the preliminary work on xanthomycin, a serial dilution type of assay was used in which *Staphylococcus aureus* H was the test organism The *S aureus* H dilution unit was adopted as the standard unit for xanthomycin It is defined as that amount of antibiotic material which, when dissolved in 1 ml of 0 75 per cent peptone and 0 25 per cent yeast extract medium at pH 7 2, will just inhibit the growth of *S aureus* H during an incubation period of 20 hours at 37° when the inoculum per 10 ml of medium is 1 drop of a 1 10 dilution of a 24 hour culture grown on the same medium

The dilution assay was very laborious and inconvenient It was useful for the preliminary work on xanthomycin, but a more convenient turbidimetric assay was developed for routine use The medium for the turbidimetric assay was peptone 0 5 per cent, beef extract 0 3 per cent, and yeast extract 0 3 per cent in distilled water, adjusted to pH 7 6 to 7 7 with NaOH 4 ml of a culture of *Staphylococcus aureus* H grown on the same medium for 16 to 20 hours at 37° were used to inoculate each 100 ml of assay medium

A stock solution of partially purified xanthomycin was assayed against *Staphylococcus aureus* H by the dilution assay method and was found to con-

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tain 10,000 units per ml This solution was stored in the refrigerator and its potency was checked frequently by the dilution assay method For the turbidimetric assay this solution was diluted with sterile distilled water to contain 100 units per ml For the standard curve 0 10, 0 08, 0 06, 0 04, and 0 02 ml of a standard solution containing 100 units per ml were pipetted in duplicate into a series of 18 × 150 mm test-tubes Similar quantities of a suitable dilution of the sample to be tested were pipetted into another series of tubes 10 ml of inoculated broth were pipetted into each tube with an automatic pipetting machine, and the tubes were then

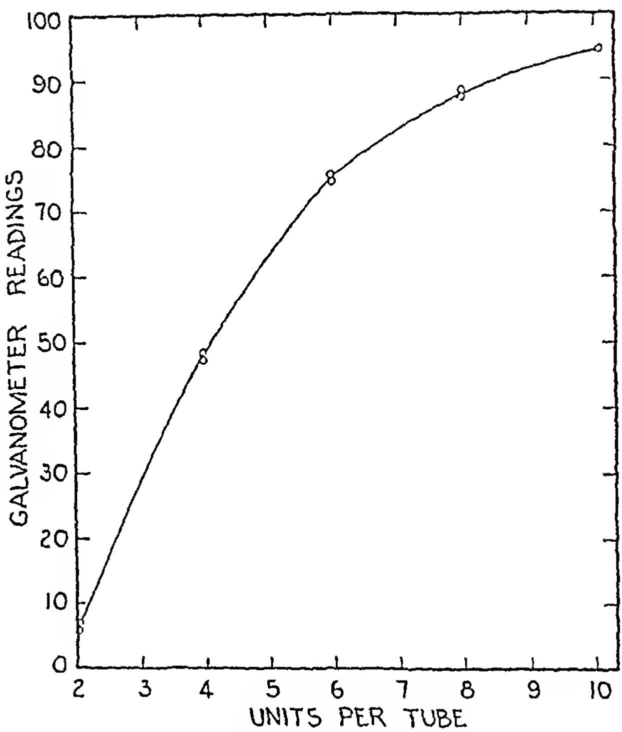

Fig 1 Standard curve for xanthomycin turbidimetric assay

incubated in a water bath at 37° for 3 hours At the end of the incubation period, growth was stopped by steaming the tubes for 10 minutes, and turbidities were read on a Lumetron model 402E colorimeter equipped with a 6500 A broad band filter A standard curve, obtained by plotting arbitrary galvanometer readings against units of the antibiotic per tube, is shown in Fig 1 Units per tube of the unknown sample were obtained from the standard curve When several levels of an unknown sample fell within the range of the standard curve, the calculated potencies usually agreed within 5 per cent, and when a sample was assayed on successive days the results usually did not vary more than 5 to 7 per cent

Fermentations in Shaken Flasks—The shaken flask technique was employed in experiments designed to find a good medium for antibiotic production Cotton-plugged 500 ml Erlenmeyer flasks containing 100 ml of medium were autoclaved 30 to 40 minutes at 15 pounds of steam pressure and the cooled flasks were inoculated with a suspension of *Streptomyces* 94 spores grown on 6 ounce bottle plates of the following medium glucose 1 per cent, asparagine 0 05 per cent, K_2HPO_4 0 05 per cent, agar 1 5 per cent, and distilled water 20 ml of spore suspension were prepared from each bottle plate and 2 to 5 ml were used to inoculate each flask of medium The inoculated flasks were incubated at 25° on a reciprocating shaker (4 inch strokes, 90 to 100 per minute), and samples were taken periodically for assay

Approximately twenty-five different media were tested The best of those tried consisted of 1 per cent each of corn steep solids, soy bean meal, dextrin (No 151 of Corn Products Refining Company), K_2HPO_4, and NaCl, and 0 1 per cent of $CaCO_3$ in tap water Yields on this medium were 5000 to 8000 units per ml with the original culture Maximum yields occurred in 4 to 5 days, after which the antibiotic concentration decreased rapidly if the fermentation was allowed to continue When corn steep solids were omitted from the medium, good growth was obtained, but no antibiotic was produced Peptone could be substituted for soy bean meal with good results, and lactose and glycerol were about as effective as dextrin

Isolation of Better Producing Strains of Streptomyces 94—*Streptomyces* 94 is susceptible to its own antibiotic Therefore an attempt was made to obtain more resistant strains and also better producers of the antibiotic

Spores of strain 94 were plated out in glucose-asparagine agar containing 6000 units of partially purified antibiotic per ml Control plates that contained no antibiotics were included The plates were incubated at 30° for 2 to 3 days after which time colonies were isolated Spore germination was apparently inhibited by the antibiotic, since many more colonies appeared on the control plates than on the plates containing the antibiotic

The new strains were tested for antibiotic production in shaken flasks and the results are shown in Table I Strain S-26 which was isolated from a control plate, did not produce any antibiotic The other strains were all isolated from plates containing the antibiotic and gave yields that were 3 to 4 times higher than those produced by the original culture

Fermentations in 30 Liter Fermenters—The 30 liter fermenters described by Peterson (1) were used for producing xanthomycin for purification studies 12 liters of medium were placed in a fermenter, 75 to 100 ml of a 2 per cent solution of octadecanol in lard oil were added as an antifoam, and the fermenter was autoclaved at 15 pounds of steam pressure for 105 minutes After cooling, each fermenter was inoculated with 500 to 700

ml of vegetative inoculum of strain 94 which had been grown 3 days on a shaker at 25° Aeration was at the rate of 0 28 cubic feet per minute and the fermenters were stirred at the rate of 400 R P M

Some of the chemical changes occurring in 30 liter fermenters were studied The medium was the same as that used in shaken flasks except that 0 5 per cent of lactose was substituted for dextrin Two of the new strains, Nos S-33 and V-28, were compared with the original culture They produced about 2 5 times as much of the antibiotic as the original strain The chemical changes brought about by the three strains were very similar The data for strain S-33 are presented in graphic form in Fig 2

TABLE I

Xanthomycin Production by New Strains of Streptomyces 94 in Shaken Flasks

Medium, corn steep solids 1 per cent, soy bean meal 1 per cent, K_2HPO_4 1 per cent, NaCl 1 per cent, lactose 0 5 per cent, $CaCO_3$ 0 1 per cent, and tap water

Strain	3 days		3 5 days		4 days		4 5 days		5 days		5 5 days	
	pH	Units per ml	pH	Units per ml	pH	Units per ml	pH	Units per ml	pH	Units per ml	pH	Units per ml
Original	7 3	0	7 5	1,380	7 4	2,450	7 4	4960	7 8	5530	8 0	<5000
	7 3		7 5	1,640	7 4	2,900	7 5	4820	7 8	5260	8 0	<5000
94-S-26	7 1	0	7 4	0	7 6	0	7 8	0				
	7 1		7 4	0	7 5	0	7 8	0				
94-S-28	7 1	4550	7 4	>10,000	7 7	12,300	7 9	4200				
	7 1		7 3	>10,000	7 6	14,900	7 9	6500				
94-V-28	7 2	>5000	7 3	>10,000	7 5	20,400	7 8	7900				
	7 2		7 4	>10,000	7 5	20,400	7 8	8400				
94-S-29	7 1	>5000	7 4	>10,000	7 6	14,900	7 9	5300				
	7 1		7 4	>10,000	7 6	12,200	7 9	4600				
94 S-33	7 1	4100	7 3	>10,000	7 5	18,200	7 8	6300				
	7 1		7 3	>10,000	7 5	18,300	7 9	6100				
94-V-33	7 1	1145	7 4	7,460	7 4	14,400	7 7	9300				
	7 2		7 2	7,800	7 4	15,700	7 7	8800				
94-V-34	7 1	>5000	7 3	>10,000	7 6	17,100	7 8	6800				
	7 1		7 3	>10,000	7 6	15,400	7 8	6000				

The antibiotic began to appear early in the fermentation and reached a maximum around 40 hours, after which it decreased rapidly The pH increased early in the fermentation, dropped as the yield of the antibiotic approached its maximum, and then rose slightly throughout the rest of the fermentation Sugar, calculated as lactose, declined throughout the fermentation Total soluble nitrogen decreased from the time of inoculation to the time of maximum yield of the antibiotic, it then began to increase, apparently owing to autolysis There was very little ammonia nitrogen in the medium at the time of inoculation and it disappeared in the first 50 hours

Analytical Methods—Kjeldahl nitrogen was determined by the micromethod of Johnson (2) and ammonia nitrogen by the method of Gailey *et al* (3), except that the ammonia was removed by steam distillation rather than by aeration For lactose the samples were autoclaved at 120° for 15 minutes in 0 75 N HCl, and reducing sugars were determined by the method of Shaffer and Somogyi (4) Reagent 50 with 5 gm of KI per liter was used and the samples were heated in a boiling water bath for 25 minutes

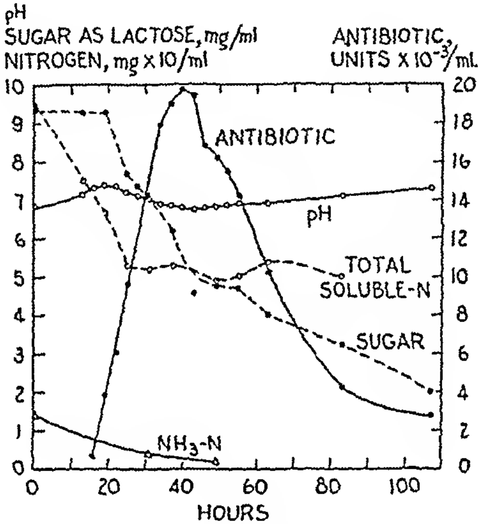

Fig 2 Chemical changes in a *Streptomyces* 94 fermentation

Purification of Xanthomycin

Two major procedures were employed in concentrating xanthomycin The active material in culture filtrates was first adsorbed on norit, from which it was eluted with 0 1 N HCl saturated with butanol After concentration of the eluate, the antibiotic was extracted into chloroform and then into acidified water It could be precipitated from concentrated water extracts with picric acid or ammonium reineckate

Adsorption of Antibiotic on Norit and Elution—The mycelium was removed by filtration and after the pH of the culture filtrate was adjusted to 7 5 to 8 0 with NaOH, 2 to 3 per cent of norit was added Usually approximately 1 per cent of Celite 545 was also added to aid in filtration Small batches were stirred for approximately 1 hour and were then filtered through a Büchner funnel Large batches were filtered through a filter press and the filtrate was pumped through three additional times to insure good ad-

sorption. Generally 75 to 95 per cent of the active material was adsorbed on the norit.

The norit was washed with a volume of water saturated with butanol equal to one-third the volume of original culture filtrate. This removed much inactive material and only little of the antibiotic. The operation was conveniently carried out in the filter press by merely pumping the solution through after the norit had been collected in the press.

The antibiotic was eluted from the norit with 0.1 N HCl saturated with butanol. This was accomplished with small batches by suspending the norit in the eluent and stirring for 30 minutes. For large batches the eluent was usually divided into two portions and each portion was pumped

TABLE II

Concentration of Xanthomycin by Norit Adsorption and Elution

	Experiment No.	Volume	Potency			Adsorbed	
		l	*units per ml*	*units per mg (dry weight)*	*total units*	*units*	*per cent*
Adsorption, culture filtrate	1	11.9	8,155	315	97,073,000	90,528,000	93
	2	33.0	5,480	180	180,840,000	142,890,000	79
						Recovery from norit	Purification (dry weight basis)
						per cent	
Elution,* norit eluate	1	5.0	16,090	945	80,450,000	89	3-fold
	2	20.0	6,310	980	126,200,000	88	5.4-fold

* The eluent was 0.1 N HCl saturated with butanol. The procedure for Experiment 1 was carried out with the usual laboratory apparatus, while a filter press was used for adsorbing and eluting in Experiment 2.

twice through the norit in the filter press. Usually 80 to 90 per cent of the antibiotic was eluted with a volume of eluent equal to one-half that of the original culture filtrate. This adsorption and elution procedure resulted in a 3- to 5-fold purification based on units per mg. of dry weight. Data for adsorption and elution of both a small and a large batch are summarized in Table II.

Extraction with Chloroform—The amount of the antibiotic extractable from aqueous solution with solvents increased as the pH was raised from 2 to 8. Chloroform and butanol were the best solvents of those tried, less effective solvents were ethyl acetate, amyl acetate, and ether.

The eluate containing the active material was concentrated by distillation *in vacuo* to a small volume (500 to 2000 ml., depending on the size of the

batch and the solids content) The pH was adjusted to 7 0 to 7 5 with NaOH, precipitated material was removed by centrifugation, and the concentrate was extracted with 2 to 3 equal volumes of chloroform From the combined chloroform extracts, the active material was extracted into a small volume of water acidified to pH 2 or lower

This extraction process brought about a 40- to 60-fold increase in purity of the antibiotic If the water-chloroform-water extraction process was repeated, the purity of the antibiotic was usually increased a few fold Data from a typical experiment are shown in Table III

Precipitation of Active Material with Picric Acid and Ammonium Reineckate—After the water extract had been concentrated to a small volume (50 ml or less) by distillation *in vacuo*, a saturated solution of picric acid in alcohol or ammonium reineckate in water was added drop by drop until a pre-

Table III

Concentration of Xanthomycin by Extraction with Chloroform and Precipitation with Picric Acid

Fraction	Quantity	Potency			Recovery	Purification (dry weight basis)
	ml	*units per ml*	*units per mg*	*total units*	*per cent*	
1 Norit eluate	3010	20,170	980	60,711,700		
2 Water chloroform-water concentrate	212	268,500	42,620	58,816,000	97	44-fold
3 Treatment in Fraction 2 repeated	225	275,500	58,620	61,875,500	102	60 fold
	mg					
4 Picrate prepared from Fraction 3	163 2		293,500	47,899,200	79	300 fold

cipitate no longer formed The use of either precipitant resulted in a 5- to 6-fold increase in purity The purest reineckate obtained from aqueous concentrates prepared by the above extraction procedure had a potency of 264,000 units per mg , and the purest picrate had a potency of 293,500 units per mg (Table III) Attempts to crystallize the derivatives which were of this order of potency were unsuccessful However, it was possible to obtain a crystalline reineckate after the aqueous concentrate had been purified further by counter-current distribution as described below

Purification by Counter-Current Distribution—The Craig (5, 6) technique of counter-current distribution was employed in purifying the active material further and separating its various components As a preliminary experiment, the distribution of the antibiotic (hydrochloride) between chloroform and buffer at various levels of pH was determined The distribution

coefficients are given in Table IV. At pH 2 0 the antibiotic was practically all retained in the buffer phase, while at pH 8 0 over 90 per cent was extracted into the chloroform

The distribution pattern obtained for an aqueous concentrate of the hydrochloride of the antibiotic in the Craig apparatus is shown in Fig 3 Chloroform and 0 1 M phosphate buffer at pH 4 40 were the two phases The lower portion of each tube in the Craig apparatus was filled with chloroform saturated with the buffer, and an equal volume, 8 ml, of buffer saturated with chloroform was added to the upper portions of Tubes 1 to 24 To the upper portion of Tube 0 were added 8 ml (500,000 units) of a solution of the antibiotic in buffer saturated with chloroform The apparatus was inverted thirty times and after the layers had separated the upper

TABLE IV

Distribution of Xanthomycin between Equal Volumes of Chloroform and 0 02 M Phosphate-Citrate Buffer*

pH	Distribution coefficient, $\frac{\text{chloroform}}{\text{buffer}}$
2 0	0 02
3 0	0 2
4 0	0 6
5 1	3 0
6 0	6 2
7 2	8 1
8 0	11 2

* The material used in this experiment was a mixture of approximately 90 per cent of xanthomycin A, 10 per cent of xanthomycin B, and 0 1 per cent of the third component as shown later by counter-current distribution studies

part of the apparatus was rotated one stage This was repeated for twenty-four transfers After the twenty-fourth transfer the buffer layer of each tube was acidified to pH 1 0 with concentrated HCl and the apparatus was again inverted a few times to extract the antibiotic into the buffer phase Samples of the buffer layer were then withdrawn for assay

The pattern (Fig 3) shows that the material consisted of at least three antibiotic components Similar patterns were always obtained for antibiotic concentrates The main component, represented by the second peak, was named xanthomycin A, and it made up 65 to 90 per cent of the total antibiotic The component represented by the first peak was named xanthomycin B and it ranged from 10 to 35 per cent of the total The material which occurred in Tubes 23 and 24 never amounted to more than 1 per cent of the total It was not named and no further work was done on it

An antibiotic concentrate containing 290 million units in 60 ml of aqueous solution was run through the Craig apparatus, 8 ml at a time The volume of the concentrate was not reduced further because the use of more concentrated solutions resulted in difficulties with emulsions Chloroform and 0 6 M phosphate buffer at pH 4 40 were the two phases After each 8 ml portion was distributed in the apparatus, the contents of each tube were withdrawn and separated The antibiotic from each chloroform portion was then extracted into an equal volume of 0 05 M phosphoric acid All the fractions from the chloroform layer and all those from the

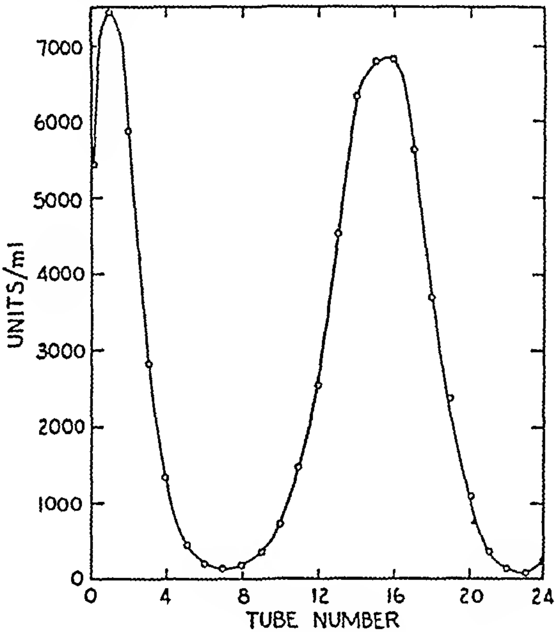

Fig 3 Distribution pattern for a xanthomycin concentrate (chloroform 0 1 M phosphate system, pH 4 40)

buffer layer were kept separate Corresponding fractions from each of the distributions were combined for assays and nitrogen determinations The results are shown in Table V Approximately 15 per cent of the total antibiotic material was present in the first peak, Tubes 0 to 6, approximately 85 per cent was present in the second peak, Tubes 7 to 23, and only 0 5 per cent was represented by the third component in Tube 24 The nitrogen figures show that nitrogen-containing impurities did not follow the same distribution pattern as the antibiotic The nitrogen figures are not absolute values but are only relative because not all the nitrogen in the antibiotic appeared as Kjeldahl nitrogen

Preparation of Crystalline Reineckate of Xanthomycin A—The fractions from the chloroform layers of Tubes 10 to 19 (Table V) were combined for preparation of the reineckate of xanthomycin A A sample of the combined fractions was run through the Craig apparatus to test for antibiotic homogeneity Chloroform and 0 3 M phosphate buffer at pH 4 25 were

TABLE V

*Craig Counter-Current Distribution Studies on Xanthomycin**

Tube No	Chloroform layer			Buffer layer		
	Nitrogen	Antibiotic		Nitrogen	Antibiotic	
	γ per ml	*units per ml*	*units per γ N*	*γ per ml*	*units per ml*	*units per γ N*
0		6,800			229,000	
1		10,300			233,000	
2		13,500			122,000	
3		17,100			60,000	
4		18,900			33,000	
5		34,900			22,000	
6		41,800			24,000	
7		54,000			29,000	
8	10 6	70,100	6,610		39,000	
9		95,900			45,400	
10	13 7	122,000	8,910	15 0	70,000	4650
11		158,000		14 5	91,000	6250
12	23 0	217,000	9,430	17 3	126,000	7300
13		261,000		20 2	162,500	8050
14	31 0	336,000	10,800	25 5	200,000	7850
15		409,000		31 8	245,000	7700
16	32 4	410,000	12,600	24 8	222,000	8950
17		371,000		25 1	197,000	7850
18	25 7	281,000	10,900	17 7	137,000	7750
19		156,000		11 8	76,000	6450
20	17 2	88,000	5,120		38,000	
21	14 0	33,400	2,390		16,000	
22		11,100			4,800	
23		9,800			2,000	
24		21,800			2,400	

Original sample, 4720 units per microgram of N

* The two phases were chloroform and 0 6 M phosphate buffer, pH 4 40

the two phases The distribution pattern is shown in Fig 4, there was only approximately 0 2 per cent of xanthomycin B present in the material

The remainder of the combined fractions was used to prepare the reineckate as follows The yellow aqueous solution was concentrated to 50 ml and a saturated solution of ammonium reineckate in water was added drop by drop until a precipitate no longer formed The precipitate which was

partially crystalline was collected and dried It weighed 170 mg and had a potency of 383,000 units per mg The reineckate was recrystallized by dissolving it in a minimum amount of 95 per cent ethanol at 50° and allowing the solution to cool slowly The recrystallized product was in the form of needles and its potency was 460,000 units per mg After a second recrystallization the potency had increased to 490,000 units per mg and it was not changed by a third recrystallization The final yield was 70 mg of long orange needles, m p 165–170° (decomposition) It was quite insoluble in cold water, soluble in warm methanol, and very soluble in acetone

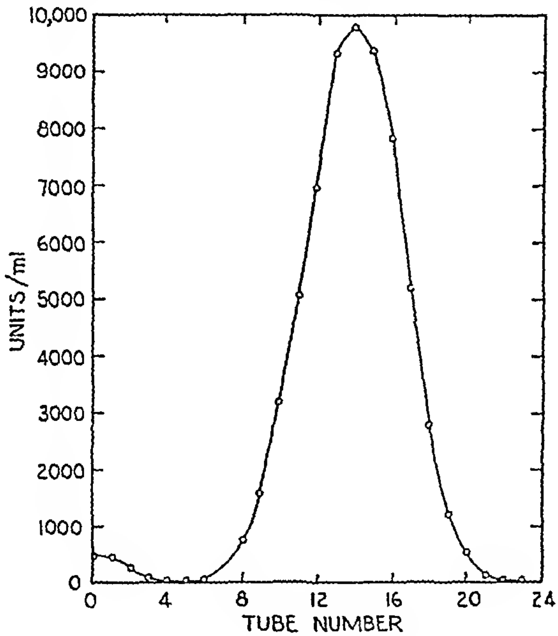

FIG 4 Distribution pattern for xanthomycin A hydrochloride (chloroform 0 3 M phosphate system, pH 4 25)

Chemical analysis (Clark Microanalytical Laboratory) gave the following figures

$C_{38}H_{57}N_{12}O_{11}S_4Cr$	Calculated	C 42 64, H 5 37, N 15 70, S 11 98
	Found	" 42 68, " 5 40, " 15 43, " 12 10

The calculated equivalent weight of the antibiotic based on the sulfur content of the Reinecke ion, which made up 30 per cent of the total weight of the reineckate, is 742 Results of diffusion rate determinations on xanthomycin A hydrochloride by the method of McBain and Liu (7) indicate that the molecular weight is the same as the equivalent weight

Another reineckate preparation of xanthomycin A was made in the same manner from the buffer fractions of Tubes 11 to 19 (Table V) After two recrystallizations from ethanol it had a potency of 490,000 units per mg and was apparently identical with the first preparation

Regeneration of Xanthomycin A Hydrochloride from Crystalline Reineckate—A 20 2 mg sample of crystalline reineckate of xanthomycin A was dissolved in 1 ml of methanol warmed to 50° A drop of concentrated HCl was added and the hydrochloride was precipitated by pouring 25 ml of anhydrous ether into the methanol solution The precipitate was removed by centrifugation and was dissolved in a few drops of anhydrous methanol, from which it was again precipitated by the addition of anhydrous ether It was then filtered, washed with anhydrous ether, and dried in a vacuum desiccator 13 mg of the hydrochloride with a potency of 565,000 units per mg were thus obtained The calculated potency of the hydrochloride, based on the potency and composition of the reineckate, is approximately 670,000 units per mg The discrepancy between the calculated and observed potencies cannot be entirely explained Although the discrepancy may be attributed in part to assay error, it suggests that there was a loss in activity upon regenerating the hydrochloride from the reineckate

Xanthomycin A hydrochloride was a yellow amorphous material very soluble in water and methanol It gave negative Sakaguchi, Molisch, ninhydrin, and ferric chloride tests The nitroprusside test for sulfur on a sample fused with sodium was negative

An absorption curve for the hydrochloride of xanthomycin A, read on a Beckman quartz spectrophotometer, is shown in Fig 5 The curve shows two maxima, with the first at 264 to 267 mμ and the second at 325 to 327 mμ

Stability of Xanthomycin—The results of stability tests on xanthomycin are shown in Table VI The antibiotic was stable in acid solution and unstable at pH 6 0 There was no loss of activity when the antibiotic stood in 3 N HCl at room temperature for 1 hour When the solutions were heated in a boiling water bath for 25 minutes, there was a 90 per cent loss of activity at pH 6 0 and only 14 per cent loss at pH 1 0

Comparison of Xanthomycins A and B—A crystalline derivative of xanthomycin B was not obtained The most pure preparations were yellow and had solubility properties similar to those of xanthomycin A Picrates of xanthomycins A and B had similar bacterial spectra and were of equal toxicity to mice

Bacterial Spectrum of Xanthomycin—A serial dilution type of assay was used in determining the bacterial spectrum of xanthomycin The medium was 0 75 per cent peptone and 0 25 per cent yeast extract in distilled water, adjusted to pH 7 2 with NaOH The test organisms were grown on the same medium for 24 hours and 1 drop of a 1 10 dilution of the cultures was

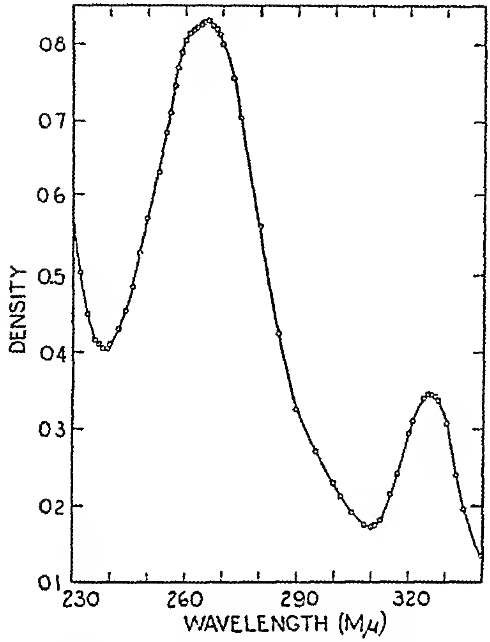

FIG 5 Absorption spectrum of xanthomycin A hydrochloride (concentration, 4 5 mg per 100 ml of water)

TABLE VI

*Stability of Xanthomycin**

Treatment	Activity lost
	per cent
pH 6 0, boiling water bath, 25 min	90
" 2 0, " " " 25 "	30
" 1 0, " " " 25 "	14
1 N HCl, " " " 25 "	23
2 " " " " " 25 "	27
3 " " " " " 25 "	45
1 " " room temperature, 60 "	0
2 " " " " 60 "	0
3 " " " " 60 "	0

* The material tested was a mixture of approximately 90 per cent of xanthomycin A, 10 per cent of xanthomycin B, and 0 1 per cent of the third component

used to inoculate each assay tube containing 9 to 10 ml of the medium After the tubes had been incubated for 16 to 20 hours, the highest dilution of the sample causing complete inhibition of growth as estimated by visual inspection was recorded

TABLE VII

Antibacterial Activity of Xanthomycin A Hydrochloride and Actinomycin

Organism	Highest dilution of inhibition*	
	Actinomycin	Xanthomycin A hydrochloride
S. aureus H	1:2.9 × 10^7 (1:4.3 × 10^7)	1:5.75 × 10^8 (1:6.05 × 10^8)
B. subtilis II	1:2.9 × 10^7 (1:4.3 × 10^7)	1:1.53 × 10^8 (1:3.0 × 10^8)
E. coli H-52	1:3.0 × 10^4 (1:6.0 × 10^4)	1:3.0 × 10^6 (1:6.0 × 10^6)
S. marcescens	1:3.0 × 10^4 (1:6.0 × 10^4)	1:6.0 × 10^6 (1:9.0 × 10^6)

* The figures in parentheses are the next highest dilutions at which growth occurred.

TABLE VIII

Bacterial Spectrum of Picrate of Xanthomycin (295,500 Units per Mg.)

Organism	Highest dilution of inhibition*
Staphylococcus aureus H	1:3.0 × 10^8 (1:4.0 × 10^8)
" *albus*	1:2.4 × 10^9 (1:3.2 × 10^9)
Micrococcus subcitreus	1:2.4 × 10^8 (1:2.8 × 10^8)
" *flavescens*	1:2.0 × 10^8 (1:2.4 × 10^8)
Escherichia coli H-52	1:2.4 × 10^6 (1:4.0 × 10^6)
Proteus vulgaris	1:2.4 × 10^6 (1:2.8 × 10^6)
Serratia marcescens	1:4.0 × 10^6 (1:8.0 × 10^6)
Aerobacter aerogenes	1:4.0 × 10^6 (1:4.0 × 10^7)
Salmonella gallinarum	1:2.4 × 10^6 (1:4.0 × 10^6)
Alcaligenes viscosus	1:4.0 × 10^7 (1:8.0 × 10^7)
Sarcina lutea	1:4.0 × 10^7 (1:6.0 × 10^7)
Bacillus subtilis I	1:2.4 × 10^8 (1:2.8 × 10^8)
" *mycoides*	1:2.8 × 10^7 (1:3.2 × 10^7)
" *mesentericus*	1:4.0 × 10^6 (1:8.0 × 10^6)
" *megatherium*	1:4.0 × 10^6 (1:4.0 × 10^7)
" *cereus*	1:2.4 × 10^6 (1:2.8 × 10^6)
" *niger*	1:2.0 × 10^8 (1:2.5 × 10^8)
" *brevis*	1:4.0 × 10^6 (1:4.0 × 10^7)
" *albolactis*	1:4.0 × 10^5 (1:4.0 × 10^6)
" *graveolens*	1:4.0 × 10^5 (1:4.0 × 10^6)
" *fusiformis*	1:8.0 × 10^7 (1:1.2 × 10^8)
" *vulgatus*	1:2.0 × 10^7 (1:4.0 × 10^7)
" *subniger*	1:4.0 × 10^7 (1:8.0 × 10^7)
Mycobacterium tuberculosis (avian)	1:2000
" " (BCG)	1:2000
" " (TB-1)	1:2000

* The figures in parentheses are the next highest dilutions at which growth occurred.

The hydrochloride of xanthomycin A, regenerated from the crystalline reineckate, and crystalline actinomycin were compared as to their activities

against four organisms The results are shown in Table VII Against *Staphylococcus aureus* H xanthomycin A was 19 times as active as actinomycin and against *Escherichia coli* it was 100 times as active

A picrate which was prepared from some antibiotic material that had not been separated into its components and which had a potency of 293,500 units per mg was tested against twenty-six organisms The results are shown in Table VIII The organisms included seventeen species of Gram-positive bacilli and micrococci, six Gram-negative species, and three strains of *Mycobacterium tuberculosis* The antibiotic was particularly active against the micrococci, the effective dilutions for inhibiting growth ranged from 1 2 0 $\times$ 10^8 for *Micrococcus flavescens* to 1 2 4 $\times$ 10^9 for *Staphylococcus albus* Gram-positive bacilli were inhibited at dilutions ranging from 1 4 0 $\times$ 10^5 for *Bacillus graveolens* to 1 2 4 $\times$ 10^8 for *Bacillus subtilis* I The material was effective against Gram-negative organisms at dilutions

TABLE IX

*Intravenous Toxicity of Xanthomycin A Hydrochloride to Mice**

No of mice	Amount injected		Total No dead at			No of survivors
			24 hrs	48 hrs	72 hrs	
	units	γ				
4	14,460	25 6	2	4	4	0
6	7,232	12 8	1	4	5	1
8	3,616	6 4	1	6	6	2
6	1,808	3 2	0	3	3	3
3	904	1 6	0	0	0	3

* Each of the mice weighed approximately 20 gm

ranging from 1 2 4 $\times$ 10^6 for *Escherichia coli* to 1 4 0 $\times$ 10^6 for *Serratia marcescens* The strains of *Mycobacterium tuberculosis* were not inhibited at dilutions as low as 1 2000

Toxicity of Xanthomycin to Mice—The results of mouse toxicity tests on xanthomycin A hydrochloride regenerated from the crystalline reineckate are shown in Table IX As little as 3 2 γ or 1808 units per 20 gm mouse was toxic The purest preparation of xanthomycin B was equally toxic when doses were expressed on the basis of units of antibiotic

Observed symptoms usually included labored breathing, lethargy, anorexia, and muscular spasms Autopsies revealed normal organs with the exception of hemorrhagic lungs

SUMMARY

Two new antibiotics have been obtained from culture filtrates of an unidentified species of *Streptomyces* The two appear to be very similar

and because of their yellow color and source they were named xanthomycin A and xanthomycin B Xanthomycin A was obtained in the form of a crystalline reineckate, but xanthomycin B did not yield a crystalline derivative Both of the antibiotics are solvent-soluble basic compounds active against Gram-positive and Gram-negative organisms, and both are extremely toxic to mice Xanthomycin A hydrochloride, regenerated from the crystalline reineckate, was toxic in amounts as low as 3 2 γ per 20 gm mouse

The authors are indebted to Professor Elizabeth McCoy and her research assistants, F R Hanson, who isolated the culture, and Barbara Sargeant, Mary A Roberts, and A P Saunders, who determined the toxicity of xanthomycin to mice They also wish to acknowledge the assistance of H E Arkens in some of the experimental work and to thank Dr S A Waksman for a sample of actinomycin

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FURTHER OBSERVATIONS ON THE LIPOTROPIC NEED FOR INOSITOL

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Inositol was originally shown to be a lipotropic agent in rats maintained under special conditions (1), the animals were depleted of fat and of B vitamins by maintenance for 3 weeks on a fat-free, high carbohydrate diet, and during a subsequent week fat synthesis was promoted by a supplement of B vitamins and a beef liver fraction Under these conditions the rats developed markedly fatty livers containing large amounts of cholesterol These fatty livers were produced despite the provision of choline, both in the liver fraction and as a supplement The accumulation of fat and cholesterol in the liver was prevented by administration of inositol The lipotropic action of inositol in rats has been confirmed by Engel (2), by Forbes (3), and by Handler (4), and has been demonstrated in humans by Abels *et al* (5)

Several explanations of the production of fatty livers resistant to choline but responsive to inositol have been offered In early work in this laboratory, crude preparations of biotin appeared to be equivalent to the beef liver fraction in producing the effect, the term "biotin fatty liver" was used to describe the fatty liver responsive to inositol Subsequent investigation with pure biotin showed that this concept was erroneous (6), the biotin explanation has been fully criticized by Best *et al* (7) In several studies in our laboratory, attempts were made to define further the dietary conditions necessary to cause fatty livers characterized by a high cholesterol content (resulting from synthesis) and by resistance to choline By means of the same basal regimen, it was found that, when thiamine was the only supplement, the fatty livers were completely responsive to choline (8) With the addition of the other B vitamins increasing resistance to choline and responsiveness to inositol were observed (8), the full effect was secured only when beef liver fraction was furnished (6) Handler (4) concluded that the beef liver fraction could be replaced by biotin plus folic acid and has also stated that the effect was due to a stimulation of food consumption

In this paper further efforts to explain the production of fatty livers showing resistance to choline and responsiveness to inositol are reported While it was found that the beef liver fraction could be partially fractionated, further work along this line appeared to be unnecessary because of results obtained with pure substances

Methods

The rats used were Wistar strain animals of both sexes reared in the colony of the Connaught Medical Research Laboratories After attaining an average weight of 90 to 100 gm , they were provided with a fat-free, high carbohydrate diet containing no source of B vitamins (9) At the end of 3 weeks, having decreased in body weight by approximately one-third, the animals were divided into strictly comparable groups of generally ten rats, except in some cases when nine were used During the subsequent week, various supplements were administered to different groups Each rat was given the basal B vitamin supplement daily by subcutaneous injection and received the following amounts thiamine hydrochloride 25 γ, riboflavin 25 γ, pyridoxine hydrochloride 40 γ, calcium pantothenate 100 γ, *p*-aminobenzoic acid 100 γ, and nicotinic acid 100 γ The additional supplements under investigation were provided orally, mixed with the food, and the dosages were as follows choline 20 mg , inositol 25 mg , folic acid 5 γ, biotin 5 γ When abnormal quantities of B vitamins were given, the following amounts were provided in the food in addition to the injected basal amounts thiamine 75 γ, riboflavin 430 γ, calcium pantothenate 2 4 mg , and nicotinic acid 6 5 mg

The beef liver fraction, similar to that used previously (10), was obtained from the Connaught Medical Research Laboratories and the procedure of Hutchings *et al* (11) was adopted in the preparation of norit eluate fractions from it Crude liver fraction was diluted, adsorption on Pfanstiehl norit A was carried out at pH 3 0, and after washing the norit with 50 per cent ethanol, it was eluated twice at 70° with ammonia-ethanol solution The combined eluates were finally concentrated *in vacuo* to the original volume of the liver fraction The filtrate obtained after separation of the norit was neutralized and concentrated to appropriate volume The daily dosage of these preparations was 2 cc per rat

At the end of the test week each animal received an intraperitoneal injection of nembutal, the livers were removed, and the crude fatty acids of the livers and carcasses were determined by methods described in previous publications (9, 12) In all cases, analyses were made on the pooled livers and pooled carcasses for each group The total cholesterol content of the petroleum ether extracts was estimated by a modification of the procedure of Schoenheimer and Sperry (13) Data for carcass fats are not included in the reported results because they are not pertinent to the discussion All data for liver lipides are results obtained by the analysis of tissues pooled for each group of animals The figures presented have been selected as typical from a much larger series of similar observations

Results

Typical effects on liver lipides caused by feeding various beef liver preparations in one of several experimental series are given in Table I Attention is drawn to the characteristic effect of the liver fraction in causing livers high in fat and in cholesterol despite the supply of choline, both in the fraction and as an additional supplement Throughout these studies, the criterion of potency has been the ability to produce fatty livers rich in cholesterol *when* choline was supplied It is apparent that the eluate fraction exhibited effects very similar to those obtained when the original material was provided The filtrate preparation contained some activity and it is obvious that adsorption had not caused complete fractionation The activity of inositol, particularly on liver cholesterol, is clearly evident

TABLE I

Effects of Beef Liver Fractions, Biotin, Folic Acid, and Abnormal Amounts of B Vitamins on Rat Liver Lipides

Supplements	Liver					
	Crude fatty acids			Cholesterol		
	mg	*per cent*	*per cent of Group I**	*mg*	*per cent*	*per cent of Group I*
Original liver fraction, choline (Group I)	2170	21 1	100	125	1 21	100
Original liver fraction, choline, inositol	456	5 8	21	25	0 33	20
Liver eluate, choline	2078	25 3	96	93	1 14	74
" filtrate, choline	1170	15 6	54	63	0 84	50
Folic acid, choline	635	11 1	29	26	0 46	21
" " biotin, choline	993	14 8	46	41	0 61	33
Additional B vitamins, choline	1303	18 3	60	49	0 70	39
" " " biotin, folic acid, choline	2185	25 7	101	94	1 10	75
Additional B vitamins, biotin, folic acid, choline, inositol	546	9 1	25	28	0 47	22

* Values in these columns are calculated by the equation,

$$\frac{\text{Average weight of liver fatty acids or cholesterol in particular group}}{\text{Average weight of liver fatty acids or cholesterol in Group I}} \times 100$$

Since Handler had reported (4) the joint efficacy of biotin and folic acid in simulating the action of the liver fraction, the effects of feeding this combination were examined The results of a typical experiment in this series are reported in Table I

It is clear that the administration of folic acid as sole replacement for liver fraction did not result in the development of fatty livers with the

characteristics of the "liver fraction fatty liver" The lipide accumulation obtained when biotin and folic acid were both provided with choline did not contain as much fat nor as high a proportion of cholesterol as was obtained with the use of liver fractions The present evidence does not substantiate Handler's conclusion (4) that the action of liver fraction in these experiments can be duplicated by administering biotin and folic acid without other additional measures

Although the basal B vitamin supplement would be expected to be adequate under most conditions, it seemed reasonable to investigate the possibility that the additional quantities of vitamins provided by the liver fraction were related to the effects obtained Consequently, the activity of further dietary supplements (equivalent to the vitamin content of the

Table II

Effects of Restricted Food Intake on Liver Lipides

Group No	Supplements* in addition to basal B vitamins	Weight gain in test week	Food consumption	Liver				
				Weight	Crude fatty acids		Cholesterol	
		gm	*gm per rat per day of test*	*gm*	*mg*	*per cent*	*mg*	*per cent*
1	Biotin, folic acid, choline	30	10 8	7 1	1073	15 1	43 6	0 61
2	Biotin, folic acid, additional B vitamins, choline	26	10 2	7 6	1675	22 2	81 0	1 07
3	" "	38	12 1	9 5	2335	24 6	117 5	1 24

* The animals were fed *ad libitum* with the exception of the members of Group 2, they were permitted only as much food as the members of Group 1 on the corresponding day of the test

liver preparation and given in detail under "Methods") of thiamine, riboflavin, calcium pantothenate, and nicotinic acid were tried The results, reported in Table I, show that feeding of additional amounts of these four factors failed to produce the same effects as the liver preparations, although the quantity of fatty acids was more similar than with other supplements However, when biotin and folic acid, as well as additional thiamine, riboflavin, pantothenate, and nicotinic acid were provided, the liver lipide responses were very similar to those observed subsequent to liver supplementation The results of one of five similar experiments are reported in Table I Even with generous provision of choline, fatty livers containing a high proportion of cholesterol were obtained The final line in Table I shows the lipotropic effect of inositol on the fatty liver produced by giving

biotin and folic acid in conjunction with abnormal amounts of other B vitamins

Under the special conditions of our experiments, the animals exhibit a distinct need for dietary inositol for lipotropic purposes Because Handler (4) has suggested that this augmented need for inositol is related to temporary stimulation of food consumption, an experiment was carried out in which the animals receiving biotin, folic acid, and the additional quantities of the other B vitamins were given only as much food as animals developing fatty livers which were much more responsive to choline, that is, animals without the augmented amounts of B vitamins The results are described in Table II A comparison of Groups 1 and 2, which had approximately the same amount of food, indicates that restriction of food consumption in Group 2 did not eliminate the effects of the additional vitamin factors on the liver lipides, but it is evident that food intake also had an effect in producing the high cholesterol fatty liver resistant to choline

DISCUSSION

Under the conditions of our experiments, fatty livers are produced in a few days by the rapid synthesis of fat, presumably from carbohydrate When thiamine is the only B vitamin supplement, the liver fat can be maintained at a normal level by supplying one lipotropic agent, choline (8) The addition of other B vitamins causes fatty livers which are not completely responsive to choline but which are amenable to choline and inositol The most marked resistance to choline was observed previously (10) when a beef liver fraction was added to the vitamin supplements, this observation was made when pure biotin and folic acid were not available The hypothesis that the liver fraction was active because of its biotin content was found to be untenable (5) The suggestion of Handler (4) that biotin and folic acid, given with customary amounts of other B vitamins, will simulate the liver fraction has not been confirmed by us The question as to whether the activity of the liver fraction is due to an unidentified constituent or to its supply of extra amounts of a number of B vitamins appears to have been settled in favor of the latter explanation and further fractionation of the liver preparation seems unnecessary All of our observations indicate that a fatty liver, at least of the type produced by *in vivo* fat synthesis, is made resistant to choline and responsive to inositol by increasing the intake of B vitamins, both in kind and in quantity

No clear explanation is available as to why liver fat can be demobilized by choline alone under some circumstances and why inositol must be provided under other conditions Presumably both substances are lipotropic because they promote the formation of phospholipides We suggested (6) previously that different fatty acids may be involved, no further informa-

tion on this point is available In every experiment in this laboratory inositol has exerted a greater effect on liver cholesterol than has choline and we have observed instances in which inositol has markedly reduced liver cholesterol without any definite effect on fatty acids Handler (4) has suggested that a large increase in food intake, with a surge in fatty acid synthesis, may be the factor causing choline resistance and inositol responsiveness Pair feeding tests, of which a typical one is reported above, show that food consumption is a contributing factor but there is also a specific effect from the B vitamin supplements

SUMMARY

Fatty livers occurring in choline-fed rats and susceptible to inosit[illegible] have been produced in animals maintained on a fat-free, high-carbohydrat[illegible] diet by supplying (a) crude beef liver fraction, (b) liver fraction eluate, [illegible] (c) biotin and folic acid with abnormal amounts of other B vitamin[illegible] Biotin and folic acid did not exhibit this effect unless the amounts of othe[illegible] B vitamins were abnormal The action of the first two supplements [illegible] apparently explained by the third The combined supplements have [illegible] specific effect which is augmented by an increased food consumption

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AN IMPROVED DITHIZONE METHOD FOR THE DETERMINATION OF SMALL QUANTITIES OF ZINC IN BLOOD AND TISSUE SAMPLES*

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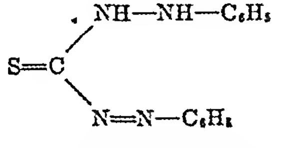
The study of zinc metabolism in experimental animals and humans is dependent upon a reliable analytical method. Numerous methods, gravimetric, turbidimetric, and colorimetric (1), have been tried and found wanting. Fischer (2) first introduced diphenylthiocarbazone (dithizone) for the analysis of metals with relation to industrial processes. This organic dye combines with a number of the heavy metals (Sandell (3)). The combination is selective for any one metal, depending upon the pH of the solution containing the metals, and the presence of salts which form complexes with other metals in solution. It was not until 1937 that Fischer and Leopoldi (4) adapted the dithizone method to the analysis of inorganic zinc. Holland and Ritchie (5) and Cowling and Miller (6) used this dye for zinc analyses in plants, and Hove *et al.* (7) in the measurement of carbonic anhydrase. The procedure employed by these authors, however, involved a preliminary extraction of other metals, principally copper, and a final extraction of zinc. Gettler (8) simplified the method by the use of a buffered solution of complex-forming salts, obviating the preliminary separation. We have further refined the method to obtain greater accuracy in the analysis of the zinc content of samples of whole blood, plasma, erythrocytes, and leucocytes and samples of tissue. Samples of the size which can be practicably obtained may contain as little as 1 to 5 γ of zinc.

Diphenylthiocarbazone has the following structural formula.



The dye is soluble in chloroform and carbon tetrachloride and insoluble in water. It decomposes in aqueous alkaline solutions. When dissolved

* The studies reported herein were supported by a grant in aid from the National Institute of Health.

in chloroform or carbon tetrachloride, the dye is dark green in transmitted and Bordeaux red in reflected light It does not fade on standing when stored in the dark at 4° When exposed to sunlight the dye is oxidized, with the production of a yellow color

At pH 5 5 and in the presence of a tartrate solution and complex-forming buffer, dithizone combines with zinc in stoichiometric proportions to form zinc dithizonate, but does not combine with other metals which may be present The completion of this reaction is accompanied by a change of color of the dithizone from green to bright red In routine extraction of samples this color change occurs slowly and appears to pass through an intermediary purple stage In our experience, this color change takes place more rapidly when the dye is dissolved in carbon tetrachloride than in chloroform

In procedures previously described, an excess of dithizone was added to insure the combination of all the zinc with dye The excess dithizone was removed from the extracted zinc dithizonate by washing with dilute ammonia This step, however, may introduce a considerable error because it is extremely difficult to determine the end-point of the washing process In the technique described, the excess dithizone is not removed The amount of zinc dithizonate present is determined by colorimetry at two critical wave-lengths, as described below

Reagents—All reagents must be absolutely zinc-free The best grade of chemically pure reagents should be obtained and, in our experience, even these may contain zinc in amounts sufficient to result in significant errors in the range of zinc content in which we are interested Reagents therefore may require purification, as described below

1 Diphenylthiocarbazone (Eastman Kodak) 100 mg are dissolved in 1000 cc of carbon tetrachloride, C P This stock solution is diluted to 1 mg per cent for the extraction of very small quantities of zinc (1 to 20 γ) This solution should be stored at 4–6° and protected from sunlight at all times No observable change in the solution occurs on standing at room temperature for a few hours

2 Buffer solution 556 gm of $Na_2S_2O_3$, C P, 90 gm of C_3COONa, C P, and 10 gm of KCN, C P, are dissolved in 1000 cc of zinc-free water The solution is then titrated with 15 N CH_3COOH to an approximate pH of 5 5, with methyl red as an indicator A final adjustment to pH 5 5 is then made with a sensitive pH meter The solution is then made up to 2000 cc with zinc-free water in a volumetric flask The buffer is then shaken with dithizone in carbon tetrachloride to remove any contaminating zinc, the extraction being repeated in a 500 cc separatory funnel until the dithizone remains a clear green

3 Tartrate solution A 20 per cent solution of $NaKC_4H_4O_6\ 4H_2O$,

C P, is made up with zinc-free water The solution is extracted with dithizone as in (2)

4 0 1 N NH_4OH, C P

5 Concentrated NH_4OH, C P

6 Methyl red indicator, 1 100 alcoholic solution

Cleaning of Glassware—Pyrex glassware must be used throughout the procedure since ordinary soft glass contains zinc Particular precautions must be observed to prevent contamination with zinc *All* glassware, including separatory funnels, transfer funnels, volumetric flasks, beakers, transfer and capillary pipettes, colorimetric tubes, test-tubes, and syringes for drawing blood, is washed with soap and water, rinsed with single distilled H_2O, and then immersed in a bath of 2 N HNO_3 for a minimum of 6 hours, preferably overnight On removal from the acid, the glassware is rinsed with zinc-free water, to which a few drops of methyl red have been added, until disappearance of the indicator's red color demonstrates the removal of all acid Satisfactory zinc-free water may be obtained by double distillation, the second of which is done in an all-Pyrex glass still Previous to use, separatory funnels are then shaken (several times if necessary) with about 20 cc of buffer solution and about 5 cc of 1 mg per cent dithizone solution, until the dithizone in the funnel remains green The dithizone is discarded An identical procedure is employed for cleaning volumetric flasks In addition, flasks are then rinsed with 0 01 N NH_4OH until yellow to methyl red This insures that the pH of any residual rinsing water will be within the range of alkalinity at which the extracted zinc dithizonate is not affected

For routine dry ashing of blood samples which contain only a total of from 1 to 20 γ of zinc, platinum crucibles must be used In this range porcelain, Vicor, Pyrex, and quartz crucibles have been found unsatisfactory, since apparently all contain minute quantities of zinc They may be used safely for tissue samples, provided the aliquots are large enough so that the error introduced is negligible Both platinum and porcelain crucibles are cleaned by boiling 2 N HCl in the vessel for at least 30 minutes, after which they are rinsed several times with zinc-free water

Method

We have routinely analyzed whole blood, erythrocyte, leucocyte, and plasma fractions thereof (9), and samples of internal organs, bone, and urine The material is placed in the crucible and slowly evaporated on a hot-plate until almost dry The crucible is then placed in an electric oven, at room temperature, and the furnace temperature raised to 600° Complete ashing requires from 12 to 24 hours

The ash is boiled in the crucible on a hot-plate with 15 to 30 cc of 2

N HCl until completely dissolved, the amount of acid used depending on the quantity of ash The dissolved ash is evaporated to a volume of about 5 cc and is then transferred to a 125 cc Squibb separatory funnel by repeated washing with small portions of hot zinc-free water When analyzing tissue samples containing more zinc than blood samples, it is desirable to bring the acid-ash solution up to 25 or 50 cc in a volumetric flask and extract a fraction thereof

2 cc of the tartrate solution, together with 2 drops of methyl red, are added to the acid-ash solution in the separatory funnel Stop-cocks are greas with silicon The contents of the funnel are then titrated with N ... H_2SO_4 to pH 5 5, at which methyl red has a peach color 50 cc of buffer are added and the contents are allowed to stand until the color has completely faded Dithizone in CCl_4 (about 10 cc of a 1 or 10 mg per cent solution, depending on the amount of zinc present) is added, and the funnel is shaken vigorously for about 2 minutes The dithizone in CCl_4 solution is allowed to collect in the bottom of the funnel, the last drop is shaken down, and the CCl_4 phase is drawn off into a 50 cc volumetric flask This procedure is repeated until the dithizone in the funnel remains a clear green The sample in the volumetric flask is brought to volume with CCl_4 Depending upon the quantity of excess dithizone present, the final color may be purple or have a greenish tinge

Colorimetry—Dithizone in CCl_4 has an absorption maximum at 620 mμ, zinc dithizonate has an absorption maximum at 520 mμ, but is transparent at 620 mμ With the filters employed, the ratio of the relative optical densities of dithizone at 620 and 520 mμ has a numerical value of 4 65 (see Fig 1)

Readings are obtained on extracted samples at both 520 and 620 mμ with the Evelyn macro photocolorimeter (10) Since the galvanometer used has an optimal accuracy within the range of from 40 to 80 per cent transmission, the final dilution is made with CCl_4 to insure readings falling within this region

The zinc content of samples is calculated from the equation

$$Z = \frac{\left(L^{520} - \frac{L^{620}}{R}\right) \times D \times K}{100} \tag{1}$$

in which Z = total zinc in micrograms in ... ure sample, L^{520} = densit (2 − log of the galvanometer reading) at 520 mμ, L^{620} = density at 620 mμ R = the ratio of density of dithizone in CCl_4 at 620 mμ and 520 mμ (r merical value determined as 4 65, see Table I), D = the dilution fa with relation to the original volume (V), K = the calibration const

(numerical value 40, see Table II), V = the volume in which all of the extracted zinc dithizonate is originally diluted

The ratio of absorption of dithizone at 620 and 520 mμ (R in equation (1)) was determined as follows A solution of dithizone in CCl_4 was prepared of such a concentration as to deflect the galvanometer to about 10 per cent of the full scale at 620 mμ A series of dilutions was then made from this so that in the lowest concentration the galvanometer registered about 80 per cent transmission The series was then read at both wave-

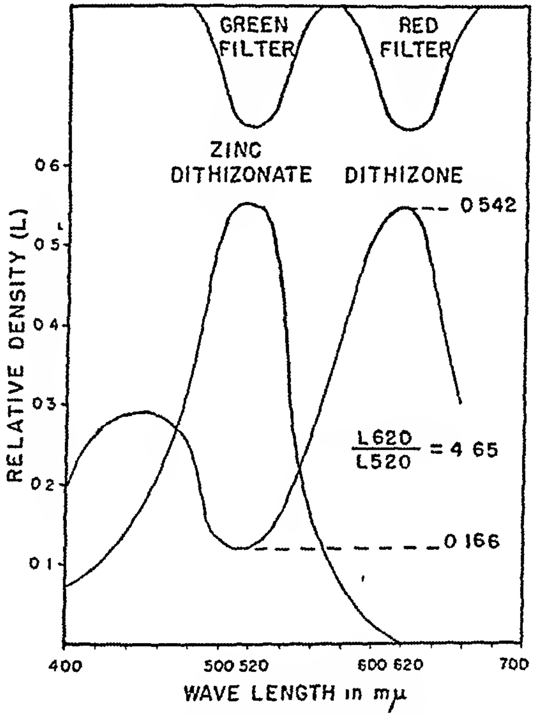


Fig 1 Absorption curves of dithizone and zinc dithizonate in carbon tetrachloride

lengths The data are given in Table I In every instance the observed value at 620 mμ was within 2 per cent of the value to be expected from the original concentration and dilution factor Values for R averaged 4 65 ± 1 per cent

Calibration—A stock solution of zinc chloride was prepared by dissolving 10 mg of metallic zinc in concentrated HCl and making up to a final olume of 1000 cc From this solution a series of standards containing om 4 to 50 γ was prepared These standards were extracted as described

above, except that they were not dry ashed The total zinc dithizonate was made up initially to 50 cc Further dilutions with CCl_4 were then made to obtain galvanometer readings within the optimal range of the instrument The data obtained are given in Table II.

TABLE I

Ratio of Absorption of Dithizone at 620 mμ and 520 mμ

Dilution factor	Galvanometer reading	L^{620} Determined	L^{620} Expected	L^{620} Deviation	L^{520}	$R = \frac{L^{620}}{L^{520}}$
per cent	*per cent*			*per cent*		
0	10 25	0 989				4 63
0 50	31 75	0 498	0 498	0	0 099	4 67
0 33	47 00	0 328	0 330	−0 6	0 072	4 60
0 33	46 00	0 337	0 330	+2 1	0 072	4 70
0 20	63 75	0 196	0 198	−1 0	0 043	4 63
0 16	69 50	0 158	0 158	0	0 034	4 63
0 10	79 25	0 101	0 099	+2 0	0 021	4 70
Average						4 65 ± 0 04

TABLE II

Calibration Constant for Zinc Dithizonate-Dithizone Solutions in CCl_4

Total Zn	Dilution factor	L^{520}	L^{620}	$\frac{L^{620}}{4\ 65}$	Corrected L^{520}	K
γ						
4	0	0 254	0 305	0 066	0 198	40 2
10	2	0 321	0 385	0 083	0 238	42 1
20	3	0 382	0 274	0 058	0 324	41 1
30	4	0 453	0 280	0 062	0 393	38 2
40	5	0 462	0 242	0 052	0 410	39 0
50	5	0 553	0 211	0 045	0 508	39 5
Average						40 0 ± 2 0

The value for K was calculated from equation (2), derived from equation (1)

$$K = \frac{2Z}{\text{Corrected } L^{520} \times D} \tag{2}$$

The numerical value of K averaged 40 0 ± 2 0, or ±5 per cent

Inasmuch as this modification of the dithizone method was designed for the analysis of the zinc content of blood samples, it was necessary to deter-

mine to what extent the accuracy of the measurement was affected by the dry ashing process

A stock solution of the $ZnCl_2$ was made up to contain approximately 1 γ of Zn per cc A series of samples, in duplicate, was prepared containing total quantities of 2, 5, 10, 15, 20, and 30 γ of zinc These were extracted without dry ashing The results are given in Table III The average value of Zn in micrograms per cc was 0 95, with a standard deviation of 0 05 This was taken as the correct concentration of the stock solution

An identical series was then dry ashed and extracted, the values obtained also being given in Table III An average value of 0 97 γ of Zn

TABLE III

Standardization of Zinc in Samples of Blood Components

Amount analyzed	Stock solution		Stock solution dry ashed		Dry ashed with white cells, 0.572 γ			Dry ashed with red cells, 3 69 γ			Dry ashed with plasma, 4 29 γ		
cc	*total* γ	γ *per cc*	*total* γ	γ *per cc*	*total* γ	*net* γ	γ *per cc*	*total* γ	*net* γ	γ *per cc*	*total* γ	*net* γ	γ *per cc*
2	1 63	0 81	1 79	0 89	2 75	2 17	1 08	6 39	2 70	1 35	6 71	2 42	1 21
2	1 76	0 88	1 77	0 88	2 31	1 74	0 87	5 53	1 83	0 91	6 11	1 82	0 91
5	4 86	0 97	5 14	1 00	5 46	4 89	0 97	8 54	4 85	0 97	8 91	4 61	0 92
5	4 81	0 96	5 08	0 99	4 92	4 35	0 87	9 02	5 33	1 06	8 68	4 39	0 88
10	9 9	0 99	9 99	0 82	10 57	10 00	1 00	12 37	8 68	0 86	14 08	9 79	0 98
10	9 88	0 98	8 20	1 06				12 59	8 90	0 88			
15	14 80	0 98	16 14	1 05				17 48	13 79	0 92	20 86	16 57	1 10
15	14 36	0 96	15 52	1 00				18 08	14 39	0 96	19 72	15 44	1 00
20	19 27	0 96	20 07	0 99	19 66	19 09	0 96	23 08	18 89	0 93	25 04	20 75	1 00
20	19 00	0 95	19 83	0 99									
30	29 18	0 97	29 88	0 99				31 36	27 67	0 95	30 08	25 79	1 29
30	29 18	0 99	30 21	1 00									
Mean*		0 95		0 97			0 96			0 98			0 93
S D , σ		±0 05		±0 069			±0 08			±0 112			±0 155

* Over all mean = 0 96, over-all S D , σ = ±0 0985

per cc , with a standard deviation of 0 07, was obtained, indicating that there was no loss of the metal in the ashing It will be noted that in both series the values for the 2 γ standards were low by about 10 per cent, averaging 0 86 γ per cc This apparent loss probably is due to slight errors in colorimetry, since similar low values were not obtained in the series described below

Three additional series were processed, in which known amounts of zinc (2 to 30 γ) were added to samples of separated canine white cells, red cells, and of plasma In each series the cells and plasma were obtained by floatation of 10 cc aliquots of the same blood Individual floatations

were done for each standard, and hence the amount of zinc due to cells and plasma was the same at all concentrations of zinc The total amount of zinc in the red and white cells and plasma was determined, in duplicate, and the average value subtracted from the total zinc found in the several standards, to obtain the net amount of zinc recovered Data are given in Table III The average net zinc recovered (in micrograms per cc) and standard deviation was 0 96 ± 0 08, 0 98 ± 0 112, and 0 93 ± 0 155 γ, in the white cell, red cell, and plasma series, respectively These values compare well with those found for the series that did not contain cells or plasma

These experiments constitute a check on the over-all accuracy of the procedure, from the separation of cells and plasma from whole blood to the final colorimetric measurement It would appear that the limit of error lies within ±5 per cent

As a check on the absence of contamination of all glassware with extraneous zinc, we have found it desirable to determine a 5 or 10 γ standard with each day's set of extractions This extraction is carried out with a random selection of crucibles, separatory funnels, volumetric flasks, pipettes, etc Results obtained in fifteen consecutive analyses of such standards show a mean value of 9 82 γ, with a standard deviation of ±0 65 The narrow limits of error found reflect the degree of prevention of contamination in all stages of the procedure

Since our investigation of zinc metabolism involves the use of the radioactive isotope of zinc, Zn^{65}, it is desirable that measurements of both total and radioactive zinc be made on the same blood or tissue sample To effect the conversion of zinc dithizonate to a water-soluble zinc salt, *all* of the extracted zinc dithizonate is returned to a clean separatory funnel, a drop of concentrated H_2SO_4 is added, followed by 10 cc of water, and the funnel is shaken until all the zinc has gone into the aqueous phase, as evidenced by the return of the carbon tetrachloride phase to the green color of dithizone We have found this method satisfactory in the analysis of blood and tissue samples in dogs (11), in normal humans (12), and in the leucemias,[1] blood dyscrasias, and various other pathological conditions

SUMMARY

A modification of the dithizone method of extracting zinc from blood and tissue samples is described The procedure permits of accurate assay of total zinc content of samples in amounts as small as 1 γ

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THE ZINC CONTENT OF NORMAL HUMAN WHOLE BLOOD, PLASMA, LEUCOCYTES, AND ERYTHROCYTES*

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The literature contains few references to the quantitative determination of zinc in human blood It is difficult to compare the scant data which are available because of the divergence of the methods used and of the units in which different authors chose to express their results (1–5)

It became apparent in the pursuit of our work on zinc in leucocytes and red blood cells, preliminary reports of which have appeared elsewhere (6–8), that the data available in the literature did not offer an adequate point of reference for our studies A technique was developed (9) for the microanalysis of zinc in biologic materials This method is sensitive for quantities as small as 1 γ (microgram) and its limits of precision are defined by a standard deviation of $\pm 10\ 0$ per cent This technique was employed throughout our work

Arc spectrography[1] of leucocytes and erythrocytes had demonstrated that, for equal numbers of cells, zinc was present in higher concentration in leucocytes than in erythrocytes We therefore decided to examine whole blood, plasma, washed erythrocytes, and washed leucocytes separately for their zinc content

Method

Venous blood samples were drawn into a 20 cc zinc-free syringe (9) which had previously been wetted with heparin Red and white blood cell counts were done in standard hemocytometers, in duplicate Only counts agreeing within 5 per cent were accepted Hematocrits were carried out in Wintrobe tubes, spinning 30 minutes at 3000 R P M Hemoglobin was determined by the method of Evelyn (10) Differential counts of leucocytes were made from smears stained with Wright's stain, 300 cells being counted

A separation of red cells, white cells, and plasma was performed by the

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[1] Through the courtesy of Dean George R Harrison and Mr Rockwell Kent, 3rd, Massachusetts Institute of Technology

flotation technique (11) All of the separated leucocytes were removed from the flotation tube and washed in a 0 02 per cent saponin solution in 0 85 per cent NaCl, until all entrained red cells had been removed, as evidenced by the absence of hemoglobin in the supernatant washing Zinc analysis was carried out on the entire mass of washed white cells

A portion of the red blood cells was transferred from the flotation tube to a centrifuge tube and washed in 0 85 N saline by centrifugation The washing was discarded, and an aliquot of the packed cells was transferred to a 4 cc hematocrit tube and made up to volume with 0 85 per cent NaCl solution The quantity of cells was such that the final hematocrit was about 30 Two further washings were performed, in the 4 cc tube, and from the third and final dilution a red blood count (in duplicate), hematocrit reading, and hemoglobin determination were obtained The duplicate red blood counts were required to check within ±5 per cent These 4 cc samples of red blood cell suspensions were the final aliquots used for analysis

The measurement of the zinc content of whole blood, plasma, leucocyte, and erythrocyte samples was performed as previously described (9) Routinely 2 cc of whole blood and 3 cc of plasma were analyzed

Calculation of Results—Zinc concentrations in whole blood and plasma were expressed in micrograms per cc analyzed Since volumetric measurement of white cells (by hematocrit) was impractical, due to the limited mass of white cells obtainable and the large variation in dimensions of the various classes of white cells, unit zinc concentrations for leucocytes were calculated in micrograms per million cells Unit zinc concentrations for red cells also were calculated in micrograms per million cells for comparison with unit white cell zinc concentrations, and in micrograms per cc of packed cells Examples of calculations of results for leucocytes and erythrocytes follow

The total number of cells in the sample of leucocytes was determined by multiplying the white cell count per c mm by the number of c mm of whole blood floated For example, the white blood cell count $= 1 \times 10^4$ cells per c mm, whole blood floated $=$ 15 cc, total cells in 15 cc of whole blood $= 1 \times 10^4 \times 1 \times 10^3 \times 1\ 5 \times 10^1 = 150 \times 10^6$ cells, total Zn in sample $= 3\ 0\ \gamma$, Zn per 1×10^6 cells $= 2 \times 10^{-2}\ \gamma$

The total number of red cells in the sample was determined by multiplying the red count of the final dilution of cells by the final volume of that suspension For example, the red blood cell count on final red cell dilution $= 3\ 5 \times 10^6$ per c mm, total cell suspension analyzed $=$ 4 0 cc, thus $3\ 5 \times 10^6 \times 1 \times 10^3 \times 4 = 14{,}000 \times 10^6$ cells, total Zn in sample $= 17\ 5\ \gamma$, Zn per 1×10^6 cells $= 1\ 25 \times 10^{-3}\ \gamma$

The zinc in cc of packed cells was calculated on the basis of micrograms

of zinc per million cells, red blood cell count, and hematocrit of whole blood, as follows the red blood cell count on whole blood (undiluted) $= 5 \times 10^6$ per c mm , hematocrit of whole blood = 45 0 per cent, thus $(1\ 25 \times 10^{-3} \times 5 \times 10^6 \times 1 \times 10^3)/(4\ 5 \times 10^{-1}) = 13\ 9\ \gamma$ per ml of packed cells

As a check on the accuracy of the individual measurements of whole blood, and the three blood components analyzed, an internal check was calculated for each blood sample The amount of zinc in 1 cc of whole blood due to the zinc content of white cells, red cells, and of plasma was calculated as follows (total Zn in all white cells analyzed)/(15 cc) = Zn in 1 cc of whole blood due to leucocytes, ((Zn)/(million red blood cells)) × ((red blood cells)/(cc of whole blood)) = Zn in 1 cc of whole blood due to erythrocytes, Zn in 1 cc of plasma × (1 − (hematocrit)/100) = Zn in 1 cc of whole blood due to plasma, = total Zn in 1 cc of whole blood

The deviation of the total whole blood zinc so calculated and the directly determined zinc content of whole blood was taken to be a measure of the over-all accuracy of the hematological procedures and of the preliminary processing, including dry ashing, as well as the chemical analyses for zinc

Material Studied—Thirty-one normal individuals, fifteen males and sixteen females, ranging in age from 18 to 45 years, were studied Repeated determinations were carried out in six of these subjects, bringing the total of single analyses to thirty-eight All blood samples were drawn in the morning with the subject fasting but not otherwise under basal conditions Each sample was analyzed for all of the data described above under "Method " In a few instances one of the components was lost in processing

In addition, five patients, with no apparent blood dyscrasias, on the medical wards of the Peter Bent Brigham Hospital, were studied One had a peptic ulcer, one had recovered from bronchopneumonia, one had milkman's disease, one was compensating from congestive heart failure, and one was suspected of having a brain tumor

Fifteen consecutive blood samples taken at 1 to 4 day intervals from the same individual, Case 53, a 22 year-old normal male, were also analyzed

Results

Unit zinc content, as described above, for whole blood and its three components for the group of normals and the patients is given in Table I, and for the series in one normal person, in Table II Also shown in Tables I to IV are the means and standard deviations (S D) for all unit zinc concentrations

The values for the amount of zinc contained in 1 cc of whole blood due to plasma, leucocytes, and erythrocytes, calculated as described above, are shown for the normals and patients in Table III, and for Case 53 in

TABLE I

Unit Values of Zinc Content of Whole Blood, Plasma, Leucocytes, and Erythrocytes

Experiment No	Date	Unit content by dithizone extraction				
		Whole blood	Plasma	Leucocytes	Erythrocytes	
Normal males						
	1947	*γ per cc*	*γ per cc*	*$\gamma \times 10^{-}$ per 1×10^{3} cells*	*$\gamma \times 10^{-8}$ per 1×10^{3} cells*	*Zn γ per cc packed red cells**
23-1	May 9	7 7	2 6	1 2	1 21	13 2
23-2	" 12	9 2	2 2	4 2	1 35	14 7
29-1	" 16	7 6	1 2	1 6	1 25	16 4
34-1	" 26	10 5	4 3	2 9	1 41	15 7
36-1	" 28	7 5	3 2	4 6	1 20	12 6
73-1	Sept 11	13 7	11 4	2 8	1 20	11 9
80-1	" 29	5 9	2 0	2 2	0 97	9 1
80-2	Oct 8	8 1	3 7	6 5	1 20	12 3
84-1	" 2	6 3	1 9	2 0	1 30	12 7
85-1	" 2	7 0	3 0	1 8	1 15	12 2
88-1	" 6	9 5	4 1	1 9	1 71	17 0
88-2	" 20	7 9	1 7	2 1	1 19	15 2
89-1	" 6	8 3	3 2	4 7	1 44	16 6
90-1	" 7	8 6	4 1	2 3	1 20	14 1
99-1	" 16	9 1	5 5	1 4	1 28	13 6
108-1	" 24	9 2	3 8	5 5	1 59	17 1
109-1	" 24	14 8	9 1	2 3	1 83	18 3
122-1	Nov 19	9 3	3 7		1 29	18 4
Mean		8 9	3 9	2 9	1 31	14 2
S D		±2 0	±2 5	±1 5	±0 25	±2 3
Normal females						
24-1	May 12	9 1	3 0	4 8	1 26	13 8
25-2	June 26	8 5	3 4	3 8	1 32	13 6
25-3	Sept 26	7 9	2 2	2 6	1 36	15 0
26-1	May 14	8 5	1 6	3 5	1 06	11 3
28-1	" 14	9 1	2 0	2 9	1 15	13 5
28-2	Oct 3	6 7	1 3	6 2	1 30	13 6
31-1	May 19	8 7	2 0	4 2	1 27	13 8
76-1	Sept 18	5 2	1 5		1 09	16 1
77-1	" 18	7 6	1 2	1 7	1 21	12 5
82-1	Oct 1		4 4	3 6	1 60	16 7
86-1	" 3		4 8	4 4	1 63	17 1
86-2	" 9	8 1	3 2	2 1	1 38	13 9
86-3	Nov 5	13 2	10 1	3 1	1 54	16 5
87-1	Oct 3		4 5	2 9	1 42	12 8
93-1	" 9	11 5	4 6	3 1	1 54	17 0

TABLE I—*Concluded*

Experiment No	Date	Unit content by dithizone extraction				
		Whole blood	Plasma	Leucocytes	Erythrocytes	
Normal females—*continued*						
	1947	*γ per cc*	*γ per cc*	*γ × 10⁻² per 1 × 10⁶ cells*	*γ × 10⁻² per 1 × 10⁶ cells*	*Zn γ per cc packed red cells**
100-1	Oct 17	7 2	2 3	3 5	1 24	11 9
101-1	" 17	8 6	3 0	4 2	1 32	14 2
115-1	Nov 4	9 4	4 2	1 9	1 17	12 9
116-1	" 5	10 4	4 1	4 6	1 85	18 4
123-1	" 19	8 4	1 2		1 52	15 5
Mean		8 6	3 1	3 5	1 37	14 5
S D		±1 8	±2 0	±1 2	±0 195	±1 9
Total normals						
Mean		8 8	3 0	3 2	1 34	14 4
S D		±2 0	±1 6	±1 3	±0 20	±2 7
Patients with no blood dyscrasias						
30-1 M	May 16	8 6	2 7	6 5	1 16	13 8
48-1 "	June 17	8 1	4 4	4 3	1 28	13 3
40-1 F	" 4	8 5	4 2	2 0	1 43	14 9
50-1 "	July 21	12 3	8 7	2 4	1 33	14 6
61-1 "	Aug 14	9 6	8 2	1 6	1 41	16 0
Mean		9 0	5 6	3 2	1 32	14 5

* Corrected for mean corpuscular volume

Table IV The total of the three values constitutes the "reconstructed" total blood zinc content

DISCUSSION

In the evaluation of the data presented above, the limitations of the various hematological techniques employed must be taken into consideration The errors inherent in the chemical analyses for zinc have been previously discussed (9)

Since Ostwald pipettes were used for the whole blood and plasma samples, little additional error was introduced in the preparation of these samples for chemical analysis

The limited quantity of leucocytes obtained from a routine sample of 15 cc of blood precluded making a volumetric measurement on these

cells It was not possible to make a final count on the leucocytes separated from heparinized blood because of clumping of cells, which persisted even after repeated washing with the saponin-saline solution The "million cell unit" obviously reflects the errors inherent in leucocyte counts carried out in standard hemocytometers, although this error was reduced by duplicate counting However, this unit allows conclusions as to the zinc content of individual white cells, and is therefore of value in the study of

TABLE II

Unit Values of Zinc Content of Whole Blood, Plasma, Leucocytes, and Erythrocytes in Normal Individual (Case 58)

Fifteen consecutive samples

Experiment No	Date	Unit content by dithizone extraction				
		Whole blood	Plasma	Leucocytes	Erythrocytes	
	1947	*γ per cc*	*γ per cc*	*γ × 10⁻² per 1 × 10⁶ cells*	*γ × 10⁻² per 1 × 10⁶ cells*	*Zn γ cc packed red cells**
1	July 31	8 4	3 3	1 2	1 48	15 7
2	Aug 4	8 3	5 2	2 2	1 37	14 5
3	" 4	9 3	6 0	1 7	1 63	17 9
4	" 5	8 7	4 4	2 0	1 43	15 0
5	" 6	7 7	2 7	4 2	1 20	11 7
6	" 7	11 1	4 2	2 0	1 77	18 7
7	" 8	9 1	3 6	5 1	1 51	15 9
9	" 12	9 9	4 8	2 0	1 40	15 1
10	" 13	8 5	2 8	2 0	1 69	16 4
11	" 14	8 4	2 8		1 66	16 5
12	" 15	10 2	5 6	1 9	1 66	17 2
13	" 18	9 4	3 6	1 5	1 14	12 5
14	" 19	9 9	2 6	3 5	1 44	16 5
17	" 22	7 6	1 5	1 9	1 19	12 5
19	" 27	9 5	5 0	3 7	1 59	17 1
Mean		9 1	3 9	2 5	1 47	15 5
S D		±0 9	±1 3	±1 1	±0 18	±0 6

* Corrected for mean corpuscular volume

clinical conditions affecting leucopoiesis, and for comparison with corresponding values for erythrocytes The development of better techniques for handling leucocytes is now in progress

The use of the million cell unit for erythrocytes also involves the same sources of error in about the same degree However, the calculation of unit zinc content of red cells on the basis of hematocrit and red count permits comparison of unit zinc concentrations in anemias and other disturbances of erythropoiesis with the normal

Inspection of the data in Tables I and II shows that the values for whole blood, plasma, leucocytes, and erythrocytes in males, females, and in the totals vary over a fairly wide range The measurements performed on one individual (Case 53) are of the same order of magnitude as found in the series of normals, though both their range and standard deviation are much narrower They occupy the central region of a hypothetical distribution curve plotted for the data obtained from the series

The variations and standard deviations of the white blood cell measurements are greater than those observed in the other categories This could be predicted from the technical limitations imposed by the processing of leucocytes, as described above Furthermore, the total zinc content of a leucocyte sample is of necessity small, because of the small number of white cells in circulation

The larger aliquots of whole blood, plasma, and red cells obtainable for routine analysis contain about 5 times as much zinc as is contained in all the white cells from 15 cc of whole blood Since the percentage error of zinc extraction tends to be constant, the absolute error will of necessity be greater for white cells than in the case of whole blood, plasma, or red cells

The distribution curves shown in Fig 1 graphically express these facts, and are plotted from data obtained in normals given in Table III The incidence is plotted for males, females, and total cases Fig 1, *A* is the curve for the directly measured whole blood zinc content The curve is symmetrical and the mode closely approximates the arithmetic mean

The distribution curves for plasma, Fig 1, *B*, for leucocytes, Fig 1, *C*, and for erythrocytes, Fig 1, *D*, are plotted, not from the directly measured unit values, but from the "reconstructed" value of zinc in 1 cc of whole blood due to the particular component given in Table III It was felt that the latter values more closely represented a fractional analysis of blood than did the direct determinations

The curve for plasma, though nearly symmetrical, appears to be slightly skewed to the left It will be noted that three of the cases fall well to the positive side of the mean There was a reasonable probability that these values may have been high due to accidental contamination with zinc but, since the evidence is not conclusive, they are included in the series The inclusion of these three cases raises the mean If these are omitted, the mean falls to 1 53 γ per ml , whereas the mode is approximated at 1 25 Thus biological significance cannot be attributed to the skewness of the curve

The curve for leucocytes is grouped around a mean value of 0 21 γ per ml , with a mode of 0 170 Here again the mean is slightly higher due to the inclusion of two cases with values much greater than the mean value plus twice the standard deviation It should be stated that the absolute quan-

tities of zinc measured in the samples are extremely small, so that losses in processing, which would not show up in samples of whole blood or of the other components, could be sufficient to account for the predominantly low values found in the majority of cases

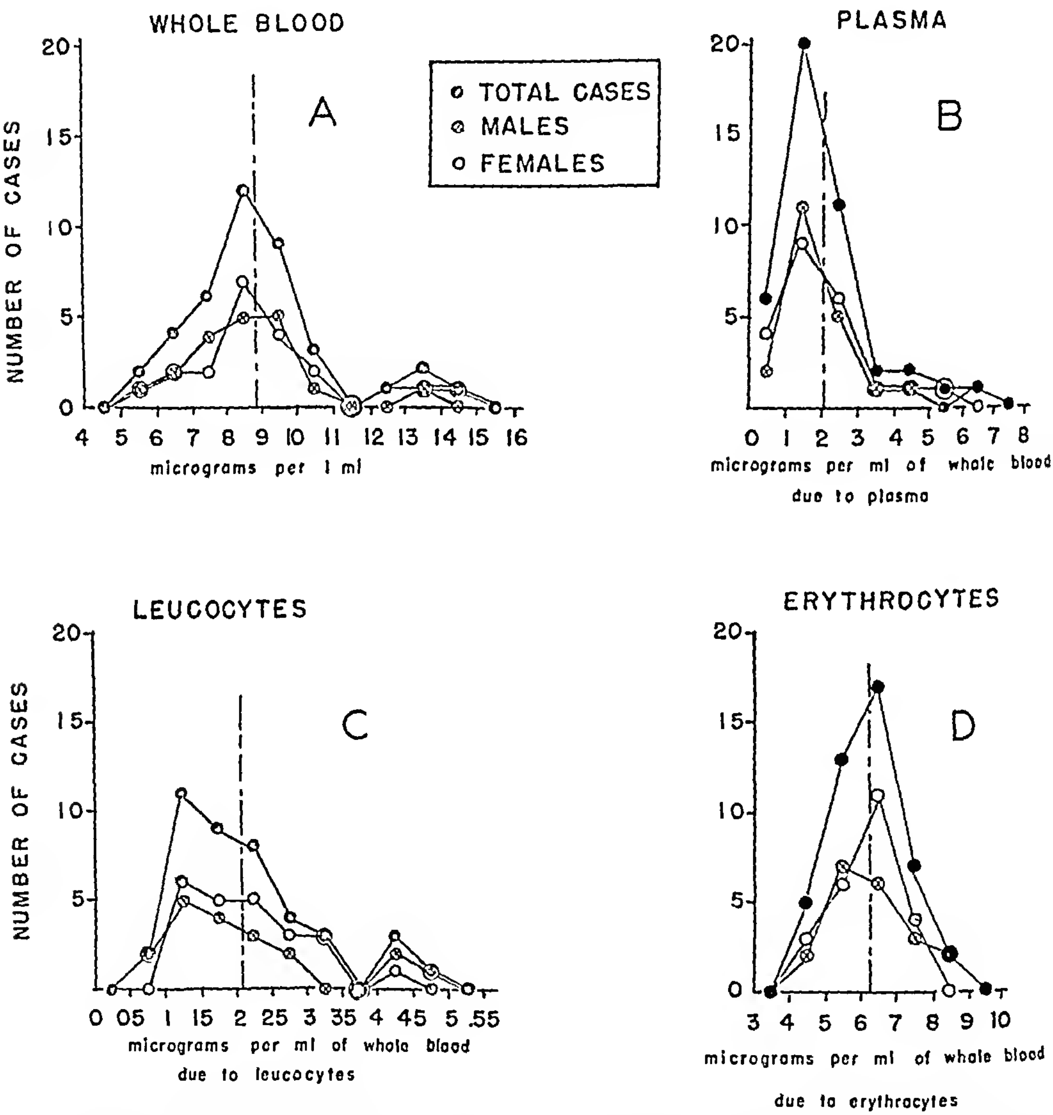

FIG 1 Distribution curves of zinc content of whole blood, plasma, leucocytes, and erythrocytes in normals

The distribution curve for red cells, Fig 1, *D*, is symmetrical and shows little skewness

It is apparent that no significant difference exists between sexes in this series

TABLE III

Zinc Content of 1 Cc of Normal Whole Blood Calculated from Unit Values of Plasma, Leucocytes, and Erythrocytes

All values in micrograms

Experiment No	Plasma	Leucocytes	Erythrocytes	Total Zn in 1 cc of whole blood		Per cent deviations
	(1)	(2)	(3)	(4)	(5)	(6)
Normal males						
23-1	1 4	0 08	6 2	7 7	7 7	0
23-2	1 2	0 33	6 8	8 4	9 2	−8 7
29-1	0 7	0 11	5 8	6 6	7 6	−13 2
34-1	2 4	0 20	6 9	9 5	10 5	−9 5
36-1	1 8	0 29	5 6	7 7	7 5	+2 7
73-1	6 2	0 22	5 5	12 0	13 7	−12 4
80 1	1 2	0 08	4 0	5 3	5 9	−10 3
80-2	2 3	0 20	4 6	7 1	8 1	−12 3
84-1	1 0	0 15	5 7	6 9	6 3	+9 5
85-1	1 5	0 14	5 9	7 5	7 0	+7 2
88-1	2 0	0 17	8 9	11 1	9 5	+16 9
88-2	0 8	0 19	7 5	8 5	7 9	+7 6
89-1	1 6	0 25	7 6	9 4	8 3	+13 3
90-1	2 3	0 25	6 4	9 0	8 6	+4 7
99-1	3 1	0 12	5 9	9 1	9 1	0
108-1	2 1	0 47	7 7	10 3	9 2	+12 0
109-1	4 9	0 15	8 4	13 5	14 8	−8 9
122-1	1 4		6 1		9 3	
Mean	2 10	0 20	6 4	8 8	8 9	−0 1
S D	±1 40	±0 095	±1 25	±2 03	±2 0	±10 0
Normal females						
24-1	1 8	0 32	5 6	7 7	9 1	−15 4
25-2	1 8	0 32	6 0	8 1	8 5	−4 7
25-3	1 2	0 18	6 6	8 0	7 9	+1 3
26-1	0 9	0 24	4 7	5 8	8 5	−31 8
28 1	1 1	0 19	6 2	7 5	9 1	−17 6
28 2	0 6	0 47	6 0	7 1	6 7	+6 0
31-1	1 1	0 23	6 0	7 3	8 7	−16 1
76-1	1 2	0 13	6 4	7 7	5 2	+48 2
77-1	0 7	0 13	5 7	6 5	7 6	−14 5
82-1	2 5	0 22	7 4	10 1		
86 1	3 1	0 22	6 1	9 4		
86-2	1 9	0 14	5 6	7 6	8 1	−6 2
86-3	6 0	0 17	6 8	13 0	13 2	−1 5
87-1	2 5	0 14	5 3	7 9		
93-1	2 5	0 26	7 8	10 6	11 5	−7 8

It should be stated that the large range of percentage deviations actually represents minute differences in the absolute quantity of zinc present in the materials measured Thus a loss or gain of 0 5 γ from a total of 5 γ is ± 10 per cent, whereas the same quantity would constitute only ± 5 per cent of the total of 10 γ This statistical treatment of the data was carried through to show the inherent limitations of the technique In our opinion, however, the method appears to be useful in clinical investigation

Normally, red blood cell zinc constitutes 75 per cent, white blood cells 3 per cent, and plasma 22 per cent of total whole blood zinc The greater portion of whole blood zinc is red cell zinc However, the individual leucocyte contains about 25 times as much zinc as the individual erythrocyte

The data available at present do not disclose the possible differences in the zinc content of the various classes of leucocytes Such studies must await the development of techniques for obtaining pure specimens of the lymphoid and myelogenous series of white cells

The findings presented suggest that zinc is a physiological constituent of blood, and its individual variations in concentration follow the mathematical pattern of commonly observed biological distribution phenomena

The normal values here reported will serve as a base for comparison of the results of studies of blood zinc levels in the blood dyscrasias, now in progress

Inasmuch as these studies employ the combined use of the chemical analyses and a radioactive tracer, Zn^{65}, the data herein reported will serve as a basis for the study of the utilization of zinc in hematopoiesis, and the transport and distribution of zinc in the normal state and in disease

SUMMARY

1 The zinc content of normal human whole blood, plasma, leucocytes, and erythrocytes in males and females has been determined

2 Red blood cell zinc constitutes 75 per cent, plasma zinc 22 per cent, and white cell zinc 3 per cent of whole blood zinc in normal human blood The individual leucocytes contain about 25 times the amount of zinc found in erythrocytes

3 Zinc concentration in blood and its components follows the distribution pattern of physiological norms

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